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UNITED STATES DEPARTMENT OF AGRICULTURE

BULLETIN No. 782

Contribution from the Bureau of Animal Industry •
JOHN R. MOHLER, Chief

Washington, D. C. PROFESSIONAL PAPER June 17, 1919

A STUDY OF THE ALKALI-FORMING BACTERIA FOUND IN MILK

By

S. HENRY AYERS, PHILIP RUPP, and WM. T. JOHNSON, Jr.,
of the Dairy Division, Bureau of Animal Industry

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HISTORICAL REVIEW.

Occasionally references to the alkali-forming bacteria are found in bacteriological literature. This group of bacteria has not received much attention, probably because under the ordinary conditions of plating their presence can not be detected, and as a consequence their rather wide distribution has been overlooked.

The fact that alkali-forming bacteria are often present in market milk in large numbers was first brought to the attention of the authors during some studies (1) [1] on the bacteriology of raw and pasteurized milk. At that time no suitable means were available for obtaining any exact figures on their numbers in market milk, so they were classed with the inert types of bacteria. Wolff (12) in a study of bacteria which developed in milk found nonliquefying short rods, colonies of which resembled those of the colon-aerogenes group. These organisms were strict aerobes which produced neither acid nor gas but turned milk alkaline without any other change. Shippen (10) also found organisms, in the milk supply of Baltimore, which gave an alkaline reaction in milk without peptonizing or coagulating it. He found that these organisms in cultural reactions closely resembled

[1] The numbers in parentheses refer to "Literature cited" at end of bulletin.

Bacillus fæcalis alkaligenes of Petruschky, also *Bacillus No. 66* described by Conn (4). *B. fæcalis alkaligenes* described by Petruschky (8) is well known as an organism which turns milk distinctly alkaline without producing any other apparent change. This is the characteristic reaction of the alkali-forming group of bacteria which separates them distinctly from other organisms which turn milk alkaline and cause peptonization at the same time.

Seiffert and Wymer (9) in 1912 reported alkali formation in litmus medium caused by bacteria which used proteins only to the smallest extent for this reaction, the chief source being citric acid and lactic acid produced by cleavage from the lactose. Both of these acids were decomposed to the alkali carbonates or bicarbonates.

Unfortunately it has been and still is the custom of most bacteriologists to consider all organisms which produce an alkaline reaction in milk as alkali-forming bacteria. It is generally assumed that the alkaline change is due to the formation of ammonia which is formed from the decomposition of the protein. Consequently when the alkali-forming bacteria are mentioned, the ammonia-producing, peptonizing types are usually brought to one's mind. Since the alkali-forming bacteria as they are considered in this paper include only those bacteria which produce an alkaline reaction due to the formation of carbonates or bicarbonates, it is necessary to define the limits of this group.

DEFINITION OF THE ALKALI-FORMING GROUP OF BACTERIA.

The alkali-forming bacteria are characterized by ability to produce an alkaline reaction in milk without visible signs of peptonization. This reaction is due to the oxidation of the salts of the organic acids present in milk to alkaline carbonates. In litmus milk the alkaline change is usually noticeable within 5 days when incubation is at 30° C. Often the change may be seen in 48 hours and occasionally a period of incubation longer than 5 days is necessary before it appears.

CAUSE OF THE ALKALINE REACTION IN MILK.

The alkaline production in milk during a period of 7 days' incubation at 30° C. is believed to be due almost entirely to formation of alkaline carbonates from the oxidation of the salts of organic acids, presumably citric acid, since fresh milk was used. This belief is based on the following facts: First, it was found that a number of the cultures tested used up all the citric acid in milk within a period of 7 days when incubated at 30° C., during which period the milk changed to reaction toward the alkaline side; and second, only a few cultures produced any ammonia in milk during a period of 7 days'

incubation at 30° C. and even then there was not a sufficient amount formed to affect the titration appreciably.

That citric acid was used up as the reaction became alkaline is shown by the results in Table 1. Since culture No. 12 was the only one which could produce even a trace of ammonia during incubation for 7 days at 30° C., it is assumed that the alkaline change of about 16 cubic centimeters tenth-normal alkali was due to the production of carbonates resulting from the oxidation of the citric acid. It may be observed, however, that in order to account for the alkaline change it would be necessary to oxidize to carbonates about one-half of the citric acid actually found to have been used up. Since the quantity of citric acid fermented, if calculated as carbonate, would be about twice as much as is necessary to account for the reduction in acidity of the milk, it seems evident that about one-half of the citric acid is oxidized to carbonates while the remainder is probably converted into other organic acids and is not carried so far as the alkaline carbonates.

TABLE 1.—*Alkaline change in milk due to oxidation of the citric acid.*

Culture No.	Titration (N/10 NaOH per 100 c. c. of milk).		Alkaline change (c. c. of N/10 alkali per 100 c. c. of milk.	Citric acid in 100 c. c. of milk.		Citric acid necessary to produce enough carbonate to account for alkaline change in milk.	Citric acid fermented but not oxidized to carbonates.
	Control.	After 7 days' incubation.		Control.	After 7 days' incubation.		
	C. c.	*C. c.*	*C. c.*	*Gram.*	*Gram.*	*Gram.*	*Gram.*
12	19.20	2.70	16.5	0.2013	0.0	0.1056	0.0957
60	19.20	3.20	16.0	.2013	0.0	.1024	.0989
130	19.20	2.60	16.6	.2013	0.0	.1063	.0950

The citric acid was determined according to the modification of Beau's (3) method. Denige's reagent was used but changed slightly as follows: To 50 grams of red oxid of mercury in 500 cubic centimeters distilled water add 110 cubic centimeters of concentrated sulphuric acid little by little and shake well until the solution is complete; dilute to 1 liter, heat to boiling, and filter when cold. The method of analysis was as follows: Place 50 cubic centimeters of milk in a 200 cubic centimeter graduated flask, add 75 cubic centimeters distilled water and 50 cubic centimeters of reagent, agitate gently, fill to mark, and mix well. Allow to settle and filter through a dry Swedish filter 12.5 centimeters No. 0 until only a faint opalescence remains. Then oxidize 100 cubic centimeters of the filtrate which corresponds to 25 cubic centimeters of milk at 75° C. by adding 1 per cent solution of potassium permanganate drop by drop until the precipitate acquires a light-brown color and settles rapidly. About

4.5 cubic centimeters of permanganate solution is sufficient. After boiling from 1 to 2 minutes the precipitate becomes almost white. If the precipitate remains colored, add from 2 to 3 drops of hydrogen peroxid solution and shake well 1 or 2 minutes. When cold, filter by weak suction in a Gooch crucible, wash with distilled water, once with alcohol, dry at 100° C., and weigh. The weight of the precipitate multiplied by 0.271 equals anhydrous citric acid. The method is accurate in milk, as shown by the fact that triplicate determinations upon a sample of milk gave the following results: No. 1, 0.2246 gram, No. 2, 0.2270 gram, and No. 3, 0.2285 gram of citric acid to 100 cubic centimeters of milk. The amount of citric acid found in the three analyses differs only by 4 milligrams.

That ammonia played little or no part in causing the alkaline reaction, especially during the first 7 days of the incubation, is shown in Table 2. Experiments were carried on to determine the amount of ammonia produced after periods of 7, 14, and 30 days of incubation at 30° C. Three sets of tubes of skim milk were inoculated with cultures of the alkali-forming bacteria and the ammonia was determined together with the reaction of the milk in each of the sets after the various periods of incubation. Each tube of milk contained 10 cubic centimeters and was marked so as to show the total volume. The amount of water lost by evaporation was made up at the time of the determination of ammonia and acidity. Five cubic centimeters or one-half of the 10 cubic centimeters was then removed for the ammonia test and the remaining 5 cubic centimeters portion was then titrated in order to determine the reaction. The ammonia was determined by the Folin method (6) with 0.2 cubic centimeter of oxyl alcohol added to prevent foaming. When the quantity of ammonia and the change in reaction of the milk was known, it was possible to calculate the amount of change in reaction due to the ammonia formed, and the difference between that and the total change was assumed to be due to formation of sodium carbonate or bicarbonate.

As may be seen from the results in Table 2, the reduction of the acidity in 58 of the 68 cultures during the first 7 days' incubation was due entirely to the formation of alkaline carbonates. In 10 cultures there was slight amount of ammonia produced, which as the results show accounted for only a very small part of the alkali produced. Generally speaking, the small amount of ammonia produced by the alkali-forming bacteria shows that the reduction of acidity in milk after 7 days at 30° C. was due almost entirely to carbonates or bicarbonates.

TABLE 2.—*Alkali production in skim milk incubated at 30° C.*

Culture No.	7 days.						14 days.						30 days.					
	C. c. N/40 H_2SO_4 neutralized by NH_3 from 5 c. c.	NH_3 milligrams in 100 c. c.	Acidity in 100 c. c.	Reduction of acidity.			C. c. N/40 H_2SO_4 neutralized by NH_3 from 5 c. c.	NH_3 milligrams in 100 c. c.	Acidity in 100 c. c.	Reduction of acidity.			C. c. N/40 H_2SO_4 neutralized by NH_3 from 5 c. c.	NH_3 milligrams in 100 c. c.	Acidity in 100 c. c.	Reduction of acidity.		
				Change from control.	Due to NH_3.	Due to Na_2CO_3 or $NaHCO_3$.				Change from control.	Due to NH_3.	Due to Na_2CO_3 or $NaHCO_3$.				Change from control.	Due to NH_3.	Due to Na_2CO_3 or $NaHCO_3$.
Control	*C. c.* 0.50	*Mg.* 4.26	*C. c.* +22.00	*C. c.*	*C. c.*	*C. c.*	*C. c.* 0.50	*Mg.* 4.26	*C. c.* +22.00	*C. c.*	*C. c.*	*C. c.*	*C. c.* 0.52	*Mg.* 4.43	*C. c.* +22.00	*C. c.*	*C. c.*	*C. c.*
10	0.68	5.79	+ 2.00	20.00	0.90	19.10	1.40	11.92	+ 6.80	15.20	4.51	10.69	2.48	21.12	+17.00	5.00	9.82	(2)
12	.53	4.51	+ 2.80	19.20	.15	19.05	1.33	11.33	+ 7.40	14.60	4.16	10.44	2.49	21.20	+15.40	6.60	9.86	(2)
31	.32	2.72	+ .20	21.80	(1)	21.80	.46	3.92	+ .40	21.60	(1)	21.60	.54	4.60	+ 1.00	21.00	.10	20.90
49	.69	5.87	+ 4.60	17.40	.95	16.45	1.40	11.92	+ 7.00	15.00	4.51	10.49	2.49	21.20	+12.80	9.26	9.86	(2)
59	.30	2.55	+ 1.00	21.00	(1)	21.00	.58	4.94	+ 1.40	20.60	.40	20.20	.87	7.41	+ 2.20	19.80	1.75	18.05
60	.42	3.58	+ 2.60	19.40	(1)	13.40	1.17	9.96	+ 2.80	19.20	3.35	15.85	1.20	10.22	+ 3.80	18.20	3.41	14.79
61	.32	2.72	+ 2.00	20.00	(1)	20.00	.58	4.94	+ 1.40	20.60	.40	20.20	.70	5.96	+ 2.40	19.60	.90	18.70
62	.15	1.28	+16.80	5.20	.....	5.20	.36	3.06	+ 1.40	20.60	(1)	20.60	.93	7.92	+ 3.20	18.80	2.05	16.75
66	.14	1.19	+17.00	5.00	.....	5.00	.23	1.96	+ 3.00	19.00	(1)	19.00	.85	7.24	+ 3.20	18.80	1.65	17.15
72	.26	2.21	+ 3.40	18.60	.....	18.60	.31	2.65	+ 3.00	19.00	(1)	19.00	.53	4.51	+ 1.00	21.00	.05	20.95
73	.22	1.87	+ 2.60	19.40	.....	19.40	.10	.85	+ 1.40	20.60	(1)	20.60	.44	3.75	+ 1.60	20.40	(1)	20.40
101	.18	1.53	+ 7.40	14.60	.....	14.60	.16	1.36	+ 1.60	20.40	(1)	20.40	.57	4.85	+ 2.60	19.40	.25	19.15
102	.11	.94	+ 7.80	14.20	.....	14.20	.18	1.53	+ 1.40	20.60	(1)	20.60	.80	6.81	+ 2.40	19.60	1.40	18.20
103	.51	4.34	+ 3.00	19.00	.05	18.95	.26	2.21	+ 1.00	21.00	(1)	21.00	.30	2.55	+ 1.00	21.00	(1)	21.00
104	.12	1.02	+ 6.40	15.60	(1)	15.60	.13	1.11	+ 1.80	20.20	(1)	20.20	.44	3.75	+ 2.20	19.80	(1)	19.80
105	(1)	(1)	+10.60	11.40	.....	11.40	.16	1.36	+ 2.80	19.20	(1)	19.20	.47	4.00	+ 2.00	20.00	(1)	20.00
106	.18	1.53	+ 3.80	18.20	.....	18.20	.55	4.68	+ 2.60	19.40	.25	19.15	.80	6.81	+ 2.00	20.00	1.40	18.60
107	.06	.51	+10.20	11.80	.....	11.80	.47	4.00	+21.00	1.00	(1)	1.00	.77	6.56	+45.20	.....	1.25	(2)
108	.13	1.11	+ 1.00	21.00	.....	21.00	.15	1.28	+ 1.60	20.40	(1)	20.40	.51	4.34	+ 1.60	20.40	(1)	20.40
111	.11	.94	+ 9.40	12.60	.....	12.60	.35	2.98	+ 1.60	20.40	(1)	20.40	.76	6.47	+ 2.20	19.80	1.20	18.60
112	.37	3.15	+ 5.60	16.40	.....	16.40	.99	8.43	+ 6.60	15.40	2.45	12.95	2.39	20.35	+10.20	11.80	9.36	2.44
113	.50	4.26	+ 3.00	19.00	.....	19.00	1.15	9.79	+ 7.80	14.20	3.25	10.95	2.08	17.71	+15.80	6.20	7.81	(2)
114	.07	.60	+ 2.20	19.80	(1)	19.80	.06	.51	+ 2.80	19.20	(1)	19.20	.24	2.04	+ 2.40	19.80	(1)	19.60
116	.36	3.15	+ 1.40	20.60	(1)	20.60	.60	5.11	+ 4.00	18.00	.50	17.50	1.20	10.22	+ 4.20	17.80	3.41	14.39
117	.07	.60	+ 5.60	16.40	(1)	16.40	.03	.26	+ 2.00	20.00	(1)	20.00	.90	7.66	+ 2.20	19.80	1.90	17.90

[1] Less NH_3 than in control.

[2] More NH_3 present than accounts for the change in acidity.

TABLE 2.—*Alkali production in skim milk incubated at 30° C.*—Continued.

Culture No.	7 days.						14 days.						30 days.					
	C. c. N/40 H_2SO_4 neutralized by NH_3 from 5 c. c.	NH_3 milligrams in 100 c. c.	Acidity in 100 c. c.	Reduction of acidity.			C. c. N/40 H_2SO_4 neutralized by NH_3 from 5 c. c.	NH_3 milligrams in 100 c. c.	Acidity in 100 c. c.	Reduction of acidity.			C. c. N/40 H_2SO_4 neutralized by NH_3 from 5 c. c.	NH_3 milligrams in 100 c. c.	Acidity in 100 c. c.	Reduction of acidity.		
				Change from control.	Due to NH_3.	Due to Na_2CO_3 or $NaHCO_3$.				Change from control.	Due to NH_3.	Due to Na_2CO_3 or $NaHCO_3$.				Change from control.	Due to NH_3	Due to Na_2CO_3 or $NaHCO_3$.
	C. c.	*Mg.*	*C. c.*	*C. c.*	*C. c.*	*C. c.*	*C. c.*	*Mg.*	*C. c.*	*C. c.*	*C. c.*	*C. c.*	*C. c.*	*Mg.*	*C. c.*	*C. c.*	*C. c.*	*C. c.*
118	0.27	2.30	+ 1.80	20.20	(1)	20.20	0.47	4.00	+ 2.00	20.00	(1)	20.00	0.61	5.19	+ 1.60	20.40	0.45	19.95
119	.12	1.02	+ 8.20	13.80	(1)	13.80	.02	.17	+ 2.60	19.40	(1)	19.40	.72	6.13	+ 3.00	19.00	1.00	18.00
120	.47	4.00	+ 5.20	16.80	(1)	16.80	1.22	10.39	+ 2.80	19.20	3.61	15.59	2.16	18.39	+ 8.40	13.60	8.21	5.39
121	.76	6.47	+ 9.20	12.80	1.30	11.50	.48	4.09	+ 6.00	16.00	(1)	16.00	.20	1.70	+ 4.00	18.00	(1)	18.00
122	.72	6.13	+ 3.00	19.00	1.10	17.90	.67	5.70	+ 2.40	19.60	.85	18.75	1.00	8.52	+ 3.20	18.80	2.41	16.39
123	.46	3.92	+ 5.20	16.80	(1)	16.80	1.13	9.60	+ 9.60	12.40	3.15	9.25	2.19	18.65	+19.00	3.00	8.36	(2)
124	.25	2.13	+ 4.00	18.00	(1)	18.00	.46	3.92	+ 3.20	18.80	(1)	18.80	.71	6.05	+ 3.80	18.20	.95	17.25
125	.05	.43	+ 2.80	19.20	(1)	19.20	.34	2.90	+ 3.60	18.40	(1)	18.40	.92	7.83	+ 3.20	18.80	2.00	16.80
126	.13	1.11	+ 4.00	18.00	(1)	18.00	.58	4.94	+ 4.60	17.40	.40	17.00	.92	7.83	+ 3.40	18.60	2.00	16.60
128	.15	1.28	+ 2.80	19.20	(1)	19.20	.20	1.70	+ 3.00	19.00	(1)	19.00	.22	1.87	+ 2.20	19.80	(1)	19.80
130	.04	.34	+ 2.40	19.60	(1)	19.60	.43	3.66	+ 2.80	19.20	(1)	19.20	.80	6.81	+ 2.60	19.40	1.40	18.00
131	.10	.85	+10.00	12.00	(1)	12.00	.14	1.19	+ 2.80	19.20	(1)	19.20	.80	6.81	+ 3.00	19.00	1.40	17.60
132	.34	2.90	+ 3.00	19.00	(1)	19.00	.77	6.56	+ 4.40	17.60	1.35	16.25	1.33	11.33	+ 5.00	17.00	4.06	12.94
133	.60	5.11	+ 5.60	16.40	.50	15.90	.43	3.66	+ 2.60	19.40	(1)	19.40	.47	4.00	+ 3.00	19.00	(1)	19.00
134	.62	5.27	+ 6.20	15.80	.59	15.21	.56	4.77	+ 5.00	17.00	.30	16.70	.50	4.26	+ 2.60	19.40	(1)	19.40
135	.16	1.36	+ 2.60	19.40	(1)	19.40	.30	2.55	+ 3.20	18.80	(1)	18.80	.61	6.19	+ 4.20	17.80	.45	17.35
136	.56	4.77	+ 4.40	17.60	.30	17.30	.72	6.13	+ 5.00	17.00	1.10	15.90	1.22	10.39	+ 5.40	16.60	3.51	13.09
137	.20	1.70	+15.00	7.00	(1)	7.00	.12	1.02	+13.60	8.40	(1)	8.40	.32	2.72	+39.60	.....	(1)	(3)
138	.37	3.15	+11.20	10.80	(1)	10.80	.47	4.00	+ 3.70	18.30	(1)	18.30	.93	7.92	+ 3.20	18.80	2.05	16.75
139	.25	2.13	+ 3.00	19.00	(1)	19.00	.65	5.53	+ 3.60	18.40	.75	17.65	1.16	9.88	+ 3.60	18.40	3.21	15.19
140	.36	3.06	+ 8.80	13.20	(1)	13.20	.08	.68	+ 3.40	18.60	(1)	18.60	.90	7.66	+ 3.60	18.40	1.90	16.50
141	.23	1.96	+13.00	9.00	(1)	9.00	.16	1.36	+ 7.20	14.80	(1)	14.80	.59	5.02	+17.80	4.20	.35	3.85
142	.18	1.53	+ 3.00	19.00	(1)	19.00	.45	3.83	+ 3.40	18.60	(1)	18.60	.81	6.90	+ 3.40	18.60	1.45	17.15
143	.27	2.30	+ 5.40	16.60	(1)	16.60	1.08	9.20	+ 6.80	15.20	2.91	12.29	2.35	20.01	+16.20	5.80	9.16	(2)
144	.27	2.30	+10.80	11.20	(1)	11.20	.12	1.02	+ 3.20	18.80	(1)	18.80	.93	7.92	+ 1.00	21.00	2.05	18.95

145	.14	1.19	+10.60	11.40	(1)	11.40	.56	4.77	+ 3.80	18.20	.30	17.90	.93	7.92	+ 2.20	19.80	2.05	17.75
146	(1)	(1)	+ 9.40	12.60	(1)	12.60	(1)	(1)	+ 2.80	19.20	(1)	19.20	.53	4.51	+ 2.40	19.60	.05	19.55
147	.38	3.24	+ 2.60	19.40	(1)	19.40	.42	3.58	+ 2.80	19.20	(1)	19.20	.87	7.41	+ 2.60	19.40	1.75	17.65
303	.21	1.79	+ 3.00	19.00	(1)	19.00	.24	2.04	+ 3.40	18.60	(1)	18.60	.34	2.90	+ .80	21.20	(1)	21.20
309	.19	1.62	+ 3.20	18.80	(1)	18.80	.33	2.81	+ 2.20	19.80	(1)	19.80	.37	3.15	+ 1.40	20.60	(1)	20.60
314	.19	1.62	+ 2.00	20.00	(1)	20.00	.21	1.79	+ 4.00	18.00	(1)	18.00	.55	4.68	+ 6.80	15.20	.15	15.05
317	.14	1.19	+ 5.80	16.20	(1)	16.20	.47	4.00	+ 1.80	20.20	(1)	20.20	.44	3.75	+ 1.60	20.40	(1)	20.40
319	.30	2.55	+ 3.20	18.80	(1)	18.80	.38	3.24	+ 1.70	20.30	(1)	20.30	.32	2.72	+ 1.80	20.20	(1)	20.20
321	.16	1.36	+ 3.00	19.00	(1)	19.00	.22	1.87	+ 2.20	19.80	(1)	19.80	.20	1.70	+ 2.00	20.00	(1)	20.00
322	.25	2.13	+ 2.20	19.80	(1)	19.80	.20	1.70	+ 1.80	20.20	(1)	20.20	.39	3.32	+ 1.60	20.40	(1)	20.40
325	.34	2.90	+ 2.40	19.60	(1)	19.60	.35	2.98	+ 2.20	19.80	(1)	19.80	.26	2.21	+ 1.60	20.40	(1)	20.40
326	.32	2.72	+ 2.20	19.80	(1)	19.80	.32	2.72	+ 1.60	20.40	(1)	20.40	.27	2.30	+ 2.20	19.80	(1)	19.80
340	.41	3.49	+ 3.40	18.60	(1)	18.60	.30	2.55	+ 1.80	20.20	(1)	20.20	.42	3.58	+ 2.00	20.00	(1)	20.00
353	.27	2.30	+15.00	7.00	(1)	7.00	.06	.51	+ 5.00	17.00	(1)	17.00	.49	4.17	+ 0.00	22.00	(1)	22.00
354	.31	2.65	+ 6.80	15.20	(1)	15.20	.63	5.36	+ 2.60	19.40	.65	18.75	.86	7.32	+ 3.00	19.00	1.70	17.30
356	.37	3.15	+ 2.60	19.40	(1)	19.40	.36	3.06	+ 1.60	20.40	(1)	20.40	.29	2.47	+ 2.40	19.60	(1)	19.60
361	.32	2.72	+ 3.00	19.00	(1)	19.00	.32	2.72	+ 1.60	20.40	(1)	20.40	.31	2.54	+ 1.20	20.80	(1)	20.80
362	.75	6.39	+ 4.40	17.60	1.25	16.35	.52	4.43	+ 1.80	20.20	.10	20.10	.34	2.90	+ 1.60	20.40	(1)	20.40

[1] Less NH_3 than in control.

[2] More NH_3 present than accounts for the change in acidity.

[3] Milk more acid than control.

The results of the 14-day incubation are similarly shown in this table. It will be noticed that the alkaline change in 46 of the 68 cultures was due entirely to the formation of alkaline carbonates and in the other 22 to the presence of carbonates and ammonia. In almost every case the reduction in acidity was due largely to carbonates and at the same time there was a considerable increase in the amount of ammonia over that produced after 7 days' incubation. Culture 107 after 14 days' incubation showed a smaller reduction of acidity than after the 7-day period, indicating that there was a secondary acid fermentation.

After 30 days' incubation the reduction in acidity in 24 cultures was due entirely to formation of carbonates or bicarbonates, there being no increase in ammonia over that contained in the control tube of skimmed milk. In 37 cultures the alkaline change was due to the combined action of carbonates and ammonia. It will be seen, however, that in most cases the greater part of the change was due to the alkaline carbonates. In general there was an increase in the quantity of ammonia, found after 30 days, over that present at the end of the 7 and 14 day incubations. Cultures 107 and 137 after 30 days' incubation were found to be more acid than the control cultures. Both these cultures showed an alkaline change after 7 and 14 days' incubation. In cultures 10, 12, 49, 113, 123, and 143 an excess of ammonia was found over that necessary to produce the alkaline change, which showed that there must have been a secondary acid fermentation. This is further substantiated by the fact that these cultures showed a smaller change in alkalinity compared with the control after the 30-day incubation than they did at the end of the 7 or 14 day incubation.

The results of the work shown in Table 2 are summarized in Table 3.

TABLE 3.—*Cultures grouped according to reaction and cause of alkali production in skim milk.*

Incubation 30° C.	Reduction of acidity due to—		Increase of acidity over control.
	Ammonia and alkaline carbonate.	Alkaline carbonate.	
Days.	*Number of cultures.*	*Number of cultures.*	*Number of cultures.*
7	10	58	0
14	22	46	0
30	[1] 37	24	2

[1] Seven ammonia and no alkaline carbonate.

This summary needs no discussion since it simply presents data which have already been discussed. These results lead to the belief

which was previously stated, namely, that the alkali-forming bacteria produce an alkaline change in milk, due primarily to the formation of carbonates or bicarbonates, resulting from the oxidation of citric acid. After a long period of incubation there is a small amount of ammonia formed by certain cultures to which may be attributed a small part of the alkaline change in reaction of milk.

A third reason to substantiate the belief that the alkaline reaction in milk produced by the alkali-forming bacteria is due to the production of alkaline carbonates from the salts of organic acids. This will be discussed in detail later. The essential point of the production of an alkaline reaction during the fermentation of the organic-acid salts and the one upon which the other adjustments of equilibrium depend is the replacement of the relatively strong organic acids by the relatively weak carbonic acid.

SOURCES OF THE ALKALI-FORMING BACTERIA.

The alkali-forming bacteria which are under discussion in this paper were isolated largely from market milk, a few being obtained from the ice cream. Milk, however, can not be considered a source of any bacteria for it is merely a conveyor in which they may grow after being introduced through contamination. While alkali-forming bacteria are commonly found in market milk they must be introduced at some stage in its history and must, therefore, have a source.

Numerous samples of soil were first examined and then plated on extract agar and incubated at 30° C. for 5 days, at which time each colony was picked off and inoculated into litmus-milk tubes. These tubes were incubated at 30° C. for 14 days, after which they were examined for the characteristic reaction produced by bacteria of the alkali group. An alkaline reaction in litmus-milk tubes after 14 days' incubation without any sign of peptonization was found to be a practically certain test for the alkali-forming group of bacteria.

All the samples of soil examined showed the presence of large numbers of alkali-forming bacteria. These organisms were found also in water from shallow and deep wells, springs, and drains. Since they are present in soil it is only natural to expect to find them in water. An examination of the bacteria on the hands of farm laborers showed as high as 12,000,000 of the alkali-forming group of bacteria, this being the approximate number distributed over both hands. The organisms in this case in all probability came from soil.

Alkali-forming bacteria were not found in the udder and teats of cows in very large numbers, since an examination of 57 samples of milk drawn into sterile tubes from 15 different cows showed their presence in only 10 cases. The highest number found in any case was 30 per cubic centimeter. These organisms were present, how-

ever, in cow feces, being found in 8 out of 14 samples examined and and they comprised about 2.7 per cent of the total bacteria. Brushings from the udders and flanks of cows also showed the presence of the organisms. Alkali-forming bacteria were also found in very large numbers in unsterilized milk pails and cans. In one examination of a 10-gallon unsterilized milk can which was about to be filled with milk, approximately 144,000,000 of these organisms were found.

From extensive studies as to the sources of the alkali-forming bacteria found in milk it is believed that they are primarily soil organisms. The fact that alkali-forming bacteria are present in soil is further supported by the work of Temple (11), who states as a result of his experiments that "It is clear that soil bacteria can use sodium or potassium salts of organic acids in such a way as to have one of the products sodium or potassium carbonate." Soil, however, can not be considered the only source of the alkali-forming group of bacteria, for there are probably many organisms from other sources which might fall into this group based on their characteristic milk reaction.

MORPHOLOGY AND GROWTH.

In the study of the alkali-forming bacteria no elaborate morphological determinations of the organisms were made. Microscopic examination of 24-hour-old agar cultures showed that of 69 cultures 6 were cocci and 62 bacilli. In individual cultures the rod-shaped organisms varied greatly in size and for that reason it was not considered advisable to subdivide this group further according to their morphological characteristics. None of the organisms were spore formers.

The optimum temperature of this group of alkali-forming bacteria isolated from milk was found to be between 20° and 30° C., and within this range they grew vigorously. There was some growth below 20° C., but only slight growth above 30° C. In fact, 8 of the cultures, when streaked on agar and incubated at 37° C. for 48 hours, showed no growth, and 1 culture, No. 354, when inoculated into milk and inoculated at 37° C., rapidly died out, so that on the ninth day upon plating 1 cubic centimeter of this milk culture, no colonies appeared on the plates. The organisms grew best under aerobic conditions, but could also develop anaerobically on certain media. No growth was observed under anaerobic conditions in a synthetic medium containing sodium-ammonium phosphate as a source of nitrogen and the salt or an organic acid as the only source of carbon. The majority of this group of organisms appeared on agar plants as a moist, white growth, but there were 6 fluorescent, 2 pink, and 1 yellow culture in the collection. The most noticeable feature of the

white growth was its consistence, about 60 per cent of the cultures producing a slimy, viscous growth that was almost impossible to remove with a needle. In litmus milk these cultures produced a typical blue coloration which usually appeared in from 3 to 5 days when incubated at 30° C. Only 3 cultures out of the 68 were found to liquefy gelatin on incubation for 45 days at 20° C. The thermal death point of the alkali-forming organisms varied from 60° to 65.6° C., when heated in milk tubes for half an hour.

SOURCES OF NITROGEN.

From preliminary results of fermentation tests with the alkali-forming bacteria, it was evident that nitrogen should be supplied in some simple form, free from carbon. It was found that sodium-ammonium phosphate could be used as a source of nitrogen. This compound gave such satisfactory results that it was used throughout the work, carbon being supplied by other substances, as carbohydrates or organic acid salts. The alkali-forming bacteria were also able to utilize nitrogen from sodium nitrate and sodium nitrite, when a suitable source of carbon was present. With these two sources of nitrogen uniform results could not be obtained, for often a culture which would grow well on one inoculation would fail to develop at another time. The more complex nitrogen-containing substances were not suitable for fermentation tests, for reasons which will be discussed. All the organisms studied grew readily with asparagin as a source of nitrogen. It was not satisfactory, however, because ammonia was liberated, which interfered with the measurement of the fermentation test substances. Similar objections also apply to the use of beef extract. This not only supplied nitrogen but carbon as well. All the cultures grew vigorously in a solution of 0.2 per cent beef extract and 0.2 per cent sodium-dibasic phosphate and produced an alkaline reaction due probably to the formation of alkaline carbonates from the organic-acid salts present in the extract. It was, therefore, quite evident that beef extract was not a suitable source of nitrogen for use in fermentation tests when a definite fermentation was to be measured.

Other nitrogen-containing substances, such as peptone, gelatin, casein, and urea were good sources of nitrogen when carbon was supplied in some other form, but as sources of both nitrogen and carbon they were unsatisfactory. In other words, they are good sources of nitrogen but poor sources of carbon.

In studying the physiology of bacteria, it is believed that the sources of nitrogen should be given considerable thought, and whenever possible a simple compound free from carbon should be selected. For the alkali-forming bacteria sodium-ammonium phosphate fulfilled this requirement.

FERMENTATION OF CARBOHYDRATES.

DEXTROSE.

In some previous work with a small number of cultures of alkali-forming bacteria, it was found by Ayers and Johnson (2) that while they apparently did not ferment sugars, an alkaline reaction was usually produced. In recent work, however, with a larger number of cultures it has been found that certain carbohydrates were fermented but that special means have to be taken to determine this action.

Extract broth is not always a suitable medium in which to determine the fermentation of dextrose, as is shown by the results in Table 4. The broth had the following composition: Dextrose 1.0 per cent, beef extract 0.4 per cent, peptone 1.0 per cent, and distilled water. The reaction was brought to the neutral point, the solution filtered, the dextrose added, and the medium sterilized. The final reaction was about plus 0.2 (Fuller's scale). The cultures were incubated at 30° C. for 7 days and a duplicate set for 21 days, and titrated at the end of each incubation period. The figures in the table represent the change in titration from the control, 10 cubic centimeters of medium being titrated with tenth-normal sodium hydrate. The fermentation was considered positive when 1 per cent or more normal acid was formed, since this is the usual amount considered to indicate fermentation. An examination of the results shows that after 7 days' incubation 15 of the cultures showed an acid fermentation in dextrose and 10 showed such a reaction after 21 days. With 13 of the cultures a higher acidity was found after 7 days than after 21 days, while 7 showed more acid after the longer incubation. After 7 days' incubation 3 of the cultures were acid but after 21 days were alkaline, while 3 other cultures were alkaline after 7 days and became acid after 21 days. It will be noted in Table 4 that 3 cultures produced gas from dextrose. These results show that the fermentation of dextrose by the alkali-forming group of organisms was very indefinite when determined in the ordinary manner by titration in the regular broth medium. Some cultures showed a distinct and others only a slight fermentation, while many produced only an alkaline reaction.

As previously noted, it was found that these bacteria produced alkaline reaction when grown in beef-extract broth without sugar. The broth contained 0.2 per cent beef extract and 0.5 per cent sodium-dibasic phosphate. While this is not exactly the same composition as that of the dextrose-extract broth, the results of titrations help to explain some of the peculiar results obtained in the dextrose fermentation, and they are incorporated in Table 4. It may be seen that all the cultures of this group gave an alkaline change in titration after 14 days' incubation in beef-extract broth;

consequently this alkali formation would interfere with the reaction as a means for determining the dextrose fermentation.

TABLE 4.—*Reaction produced by the alkali-forming bacteria in dextrose and plain-extract broth.*

Culture No.	Dextrose-extract broth.		Beef-extract broth, 14 days.	Culture No.	Dextrose-extract broth.		Beef-extract broth, 14 days.
	7 days.	21 days.			7 days.	21 days.	
	C. c.	*C. c.*	*C. c.*		*C. c*	*C. c.*	*C. c.*
10	+2.40	+1.46	−0.39	128	+1.40	−0.86	−0.37
12	+ .66	+1.40	− .43	130	− .84	− .92	− .39
31	− .04	+ .82	− .29	131	− .56	− .90	− .38
49	+2.40	+ .78	− .41	132	− .76	− .82	− .32
59	+1.60	− .96	− .37	133	[2]+5.36	[2]+2.72	[2]− .40
60	−1.00	− .84	− .38	134	[2]+4.44	[2]+4.76	[2]− .35
61	− .64	− .88	− .40	135	− .88	− .92	− .39
62	− .94	− .86	− .36	136	− .76	− .82	− .37
66	− .90	− .70	− .30	137	+1.12	+2.88	− .39
72	− .06	− .20	− .30	138	− .88	− .98	− .32
73	− .60	− .74	− .30	139	− .86	− .94	− .42
101	− .94	− .84	− .29	140	−1.00	− .80	− .29
102	− .88	− .90	− .30	141	+2.56	+ .76	− .62
103	− .58	− .80	− .28	142	− .80	− .86	− .25
104	− .86	− .74	− .38	143	+ .93	+ .44	− .33
105	− .94	− .84	− .32	144	− .60	− .84	− .34
106	− .80	− .88	− .42	145	− .50	−1.06	− .39
107	+1.10	+2.46	− .41	146	− .84	− .96	− .39
108	− .74	− .80	− .41	147	−1.00	− .88	− .32
111	− .86	− .90	− .40	303	− .72	− .76	− .32
112	+3.00	+ .76	− .49	309	− .26	− .86	− .23
113	+2.08	+1.58	− .40	314	− .20	+ .94	− .47
114	− .80	− .82	− .29	317	− .52	− .76	− .32
116	− .82	− .76	− .31	319	+1.54	− .48	− .44
117	− .50	− .94	− .39	321	− .44	− .20	− .42
118	− .90	− .96	− .35	322	− .30	+1.66	− .41
119	− .80	− .82	− .39	325	− .50	− .16	− .44
120	+1.94	+ .42	− .42	326	− .40	− .32	− .38
121	[2]+5.60	[2]+4.46	− .28	340	− .90	− .70	− .31
122	− .70	− .96	− .38	353	− .70	− .80	− .53
123	+1.86	+1.70	− .37	354	− .84	− .84	− .38
124	− .86	− .94	− .32	356	− .50	− .52	− .35
125	− .64	− .90	− .34	361	− .16	.....	− .43
126	− .64	− .80	− .34	362	− .40	− .34	− .37

Minus (−) signs indicate an alkaline change[1] in titration.
Plus (+) signs indicate an acid change in titration.

[1] Change from control, in c. c. N/10 NaOH titrating 10 c. c. of medium. [2] Produced gas.

In order to avoid the complicating alkaline fermentation in extract broth, synthetic media were prepared which consisted of sodium-ammonium phosphate as the only source of nitrogen with dextrose as the only source of carbon. The media used had the following composition:

		Medium A.	Medium B.	Medium C.
Sodium-ammonium phosphate	grams	2.0	1.5	1.0
Dextrose	do	10.0	3.0	10.
Potassium chlorid	do	.1	.1	.2
Distilled water	cubic centimeter	1,000	1,000	1,000

The results of the fermentation of the dextrose in these media are shown in Table 5. The change in the hydrogen-ion concentration during 14 days' incubation was used as a measure of the fermenta-

tion. It will be noted that in medium A, 25 cultures showed a distinct fermentation of dextrose, the hydrogen-ion concentration in most cases going from P_H 6.9 (control) to P_H 4.5, a few cultures reached about P_H 6.0, and only 19 cultures changed from P_H 6.9 to P_H 6.5 or lower. These cultures it was believed fermented dextrose but were called plus or minus.

TABLE 5.—*Results of fermentation of dextrose in synthetic media with different buffer contents.*

Culture No.	Medium A.		Medium B.		Medium C.		Summary of dextrose fermentation.	Culture No.	Medium A.		Medium B.		Medium C.		Summary of dextrose fermentation.
	P_H.	Dextrose fermentation.	P_H.	Dextrose fermentation.	P_H.	Dextrose fermentation.			P_H.	Dextrose fermentation.	P_H.	Dextrose fermentation.	P_H.	Dextrose fermentation.	
Control..	6.9	.....	7.2	.....	6.8	.....	.....	Control..	6.9	.....	7.2	.....	6.8	.....	.....
10	4.5	+	5.5	+	.....	.....	+	130	6.9	−	7.2	−	.....	.....	−
12	4.5	+	5.4	+	.....	.....	+	131	6.6	±	7.2	−	5.3	+	+
31	5.7	+	6.2	+	.....	.....	+	132	6.6	±	7.2	−	5.0	+	+
49	4.5	+	5.4	+	.....	.....	+	133	4.5	+	6.2	+	.....	.....	+
59	6.9	−	7.2	−	.....	.....	−	134	4.5	+	6.2	+	.....	.....	+
60	6.9	−	7.2	−	.....	.....	−	135	6.7	±	7.2	−	5.2	+	+
61	6.9	−	7.2	−	.....	.....	−	136	6.5	±	7.2	−	5.0	+	+
62	6.9	−	7.2	−	.....	.....	−	137	4.5	±	5.0	+	.....	.....	+
66	6.9	−	7.2	−	.....	.....	−	138	6.6	±	7.2	−	5.3	+	+
72	5.8	+	6.2	+	.....	.....	+	139	6.9	−	7.2	−	.....	.....	−
73	6.9	−	7.2	−	.....	.....	−	140	6.5	±	7.2	−	5.0	+	+
101	6.6	±	7.2	−	4.9	+	+	141	4.5	+	6.0	+	.....	.....	+
102	6.6	±	7.2	−	5.3	+	+	142	6.9	−	7.2	−	.....	.....	−
103	6.9	−	7.2	−	.....	.....	−	143	4.5	+	5.4	+	.....	.....	+
104	6.7	±	7.2	−	5.8	+	+	144	6.6	±	7.2	−	5.0	+	+
105	6.6	±	7.2	−	5.4	+	+	145	6.9	−	7.2	−	.....	.....	−
106	6.9	−	7.2	−	.....	.....	−	146	6.6	±	7.2	−	5.0	+	+
107	4.5	+	5.5	+	.....	.....	+	147	6.9	−	7.2	−	.....	.....	−
108	6.5	±	7.2	−	4.8	+	+	303	6.9	−	7.2	−	.....	.....	−
111	6.9	−	7.2	−	.....	.....	−	309	6.9	−	7.2	−	.....	.....	−
112	4.5	+	6.0	+	.....	.....	+	314	5.6	+	6.3	+	.....	.....	+
113	4.5	+	5.5	+	.....	.....	+	317	6.9	−	7.2	−	.....	.....	−
114	6.9	−	7.2	−	.....	.....	−	319	6.2	+	6.5	+	5.2	+	+
116	6.7	±	7.2	−	6.3	+	+	321	6.2	+	6.3	+	5.3	+	+
117	6.6	±	7.2	−	5.0	+	+	322	6.2	+	6.4	+	5.3	+	+
118	6.9	−	7.2	−	.....	.....	−	325	6.2	+	6.5	+	5.2	+	+
119	6.7	±	7.2	−	5.9	+	+	326	6.2	+	6.5	+	5.2	+	+
120	4.5	+	5.5	+	.....	.....	+	340	6.9	−	7.2	−	.....	.....	−
121	4.5	+	6.2	+	.....	.....	+	353	6.9	−	7.2	−	.....	.....	−
122	6.9	−	7.2	−	.....	.....	−	354	6.9	−	7.2	−	.....	.....	−
123	4.5	+	4.5	+	.....	.....	+	356	6.2	+	6.5	+	5.0	+	+
124	6.7	±	7.2	−	5.0	+	+	361	6.0	+	6.4	+	.....	.....	+
125	6.5	±	7.2	−	5.0	+	+	362	6.3	+	6.2	+	5.0	+	+
126	6.6	±	7.2	−	5.0	+	+								
128	6.9	−	7.2	−	.....	.....	+								

Medium B, while having less buffer than medium A, contained also less dextrose, and in it only 25 cultures showed a positive fermentation. Medium C was then tried and it contained the same amount of dextrose as medium A but half the sodium-ammonium phosphate. As this medium had considerably less buffer than medium A, it was expected that the cultures showing only a slight change in P_H in medium A would show a much larger change in medium C. This was found to be the case, as is shown in the table.

Only the cultures which showed a slight P_H change in medium A were run in medium C. From the results of the work it was evident that 44 of the 68 cultures of alkali-forming bacteria could ferment dextrose, but many of them to only a slight extent. The fermentation was slow with certain of the bacteria, as is seen in Table 6, which shows the P_H value of 3 cultures in medium A. These cultures represent 3 types of fermentation. Culture 12 reached P_H 4.5 after 3 days' incubation at 30° C., while culture 319 reached P_H 6.0 only after 10 days, and its highest hydrogen-ion concentration was P_H 5.5. Culture 140 reached P_H 6.5 in 10 days, which seemed to be its highest point, and did not show further change after 21 days' incubation.

TABLE 6.—*Three types of dextrose fermentations as shown by the hydrogen-ion concentration in terms of* PH.

Culture No.	Days.								
	1	2	3	4	5	7	10	14	21
Control	7.1	7.0	7.0	7.0	7.0	7.0	7.0	7.0	6.9
12	6.6	5.6	4.5	4.5	4.5	4.5	4.5	4.5	4.5
319	6.9	6.8	6.8	6.8	6.6	6.6	6.0	6 0	5.5
140	7.0	7.0	7.0	6.9	6.9	6.8	6.5	6.5	6.5

TABLE 7.—*Comparison of the fermentation of dextrose in an extract broth and a synthetic medium.*

Culture No.	Dextrose broth.		Dextrose-sodium-ammonium phosphate.	Culture No.	Dextrose broth.		Dextrose-sodium-ammonium phosphate.	Culture No.	Dextrose broth.		Dextrose-sodium-ammonium phosphate.
	7 days.	21 days.	14 days.		7 days.	21 days.	14 days.		7 days.	21 days.	14 days.
10	+	+	+	118	−	−	0	145	−	−	0
12	−	+	+	119	−	−	+	146	−	−	+
31	−	−	+	120	+	−	+	147	−	−	0
49	+	−	+	121	+	+	+	303	−	−	0
59	+	−	0	122	−	−	0	309	−	−	0
60	−	−	0	123	+	+	+	314	−	−	+
61	−	−	0	124	−	−	+	317	−	−	0
62	−	−	0	125	−	−	+	319	+	−	+
66	−	−	0	126	−	−	+	321	−	−	+
72	−	−	+	128	+	−	0	322	−	+	+
73	−	−	0	130	−	−	0	325	−	−	+
101	−	−	+	131	−	−	+	326	−	−	+
102	−	−	+	132	−	−	+	340	−	−	0
103	−	−	0	133	+	+	+	353	−	−	0
104	−	−	+	134	+	+	+	354	−	−	0
105	−	−	+	135	−	−	+	356	−	−	+
106	−	−	0	136	−	−	+	361	−	0	+
107	+	+	+	137	+	+	+	362	−	−	+
108	−	−	+	138	−	−	+				
111	−	−	0	139	−	−	0				
112	+	−	+	140	−	−	+				
113	+	+	+	141	+	−	+				
114	−	−	0	142	−	−	0				
116	−	−	+	143	−	−	+				
117	−	−	+	144	−	−	+				

Minus (−)=alkaline. Plus (+)=acid. Zero (0)=no change.

The results of the fermentation of dextrose in these simple synthetic media were so different from the fermentation in the complex extract-broth medium that the results are compared in Table 7. In the dextrose-broth medium incubated for 7 days, 15 cultures showed a production of at least 1 per cent normal acid and on this basis 10 cultures showed a dextrose fermentation in the same medium after 21 days' incubation, yet in the synthetic medium 44 of the 68 cultures fermented dextrose. The results indicate very clearly the inaccuracies which may occur in the study of the fermentation of test substances in complex media.

GALACTOSE.

The alkali-forming bacteria fermented galactose in a manner nearly identical with dextrose. Practically all the cultures which fermented dextrose fermented galactose and, in fact, reached about the same hydrogen-ion concentration in the synthetic medium.

LACTOSE, SACCHAROSE, AND RAFFINOSE.

Lactose is not fermented in the ordinary beef-extract medium when measured by the usual titration methods or by the change in hydrogen-ion concentration. The assumption that the alkali-forming bacteria are nonlactose fermenters based on results of the fermentation of the sugar in ordinary media is quite incorrect, as is shown in Table 8. It was found that 11 of the cultures showed a slight change in hydrogen-ion concentration in synthetic medium A. Medium A and medium C were the same as previously described except that lactose was substituted for dextrose. The slight fermentation of lactose was more clearly indicated by the results obtained with medium C. There is included also in the table the reaction of skim-milk cultures of the organisms after 7, 14, and 30 days' incubation at 30° C. In every case the acidity decreased during the first 7 days and then increased. After 14 days the acidity in all but 3 cultures was higher than on the seventh day, while in 30 days all the cultures showed a marked increase in acidity compared with that of the seventh day.

The acid formation, which is probably secondary in milk, correlates perfectly with the cultures which showed a slight fermentation of lactose in the synthetic media. All the cultures which showed no indication of fermentation of lactose in the sodium-ammonium-phosphate medium failed to show any secondary acid formation in milk during a period of 30 days.

Saccharose was found to be fermented by only 2 of the cultures, Nos. 31 and 72. In a synthetic medium these change the reaction from P_H 7.1 to P_H 6.5 and 6.6, respectively. This indicates only a slight fermentation.

Raffinose was not fermented by any of the cultures even when several of the different media were used.

TABLE 8.—*The cultures which fermented lactose in synthetic media and the milk reactions by titration of the same cultures.*

Culture No.	Lactose synthetic media.		Reaction of milk[1] cultures after—		
	A.	C.	7 days.	14 days.	30 days.
	P_H.	P_H.	*C. c.*	*C. c.*	*C. c.*
Control	6.9	6.8	22.0	22.0	22.0
10	6.7	6.1	2.0	10.6	17.0
12	6.7	6.1	2.8	7.4	15.4
49	6.7	6.1	4.6	7.0	12.8
107	6.7	4.8	10.2	21.0	45.2
112	6.7	6.4	5.6	6.6	10.2
113	6.7	6.4	3.0	7.8	15.8
120	6.6	6.4	5.2	2.8	8.4
123	6.7	6.0	5.2	9.6	19.0
137	6.6	5.9	15.0	13.6	39.6
141	6.7	6.3	13.0	7.2	17.8
143	6.7	6.1	5.4	6.8	16.2

[1] 100 c. c. of milk titrated with tenth-normal NaOH.

In the study of the fermentation of sugars by the alkali-forming bacteria care must be exercised to use a medium in which organic-acid salts are not present, for they are readily oxidized to alkaline carbonates, which may mask an acid fermentation of the sugar. A synthetic medium is of the greatest assistance in this connection.

FERMENTATION OF ALCOHOLS.

In a study of the fermentation of sugars it is customary to include some of the polyatomic alcohols, such as adonite, dulcite, mannite, and glycerin. It was found that these polyatomic alcohols were not particularly good sources of carbon for the alkali group of organisms when used in a synthetic medium composed of sodium-ammonium phosphate 2 grams, potassium chlorid 0.1 gram, test alcohol 2 grams, and distilled water 1,000 cubic centimeters. None of the cultures fermented adonite or dulcite, but, as is shown in Table 9, 5 cultures fermented mannite and 3 fermented glycerin. In mannite the maximum change in hydrogen-ion concentration was from P_H 7.1 (control) to P_H 6.1 and in glycerin from P_H 6.9 (control) to P_H 6.4.

The monoatomic alcohols are not commonly used as test fermentation substances, but it was thought advisable to determine whether some of them could supply carbon for the alkali-forming bacteria. Four of the monoatomic alcohols, namely, methyl, ethyl, propyl, and amyl, were tested with different amounts in the sodium-ammonium phosphate medium just described. As nothing was known regarding the effect of different concentrations of these alcohols on the bacteria, 3 different sets of media with 10, 5, and 3 grams, respectively, of alcohol to the liter were used. The cultures were incubated 7 days at 30° C. and any change over P_H 0.2 in a hydrogen-ion

concentration was considered a positive fermentation. Methyl alcohol was not fermented by any of the cultures, which may be due to the poisonous character of this alcohol. As may be seen from Table 9, the majority of the cultures fermented propyl alcohol. Forty-eight of the 68 cultures fermented both propyl and ethyl alcohol when 5 grams of alcohol per liter was used in the medium. It will be noted that with the other quantities of alcohol more cultures fermented the propyl than the ethyl alcohol. Amyl alcohol was not so readily fermented.

In ethyl alcohol the maximum hydrogen-ion change was from P_H 7.1 (control) to P_H 5.7, in propyl alcohol from P_H 7.0 (control) to P_H 5.4, and in amyl alcohol from P_H 6.9 (control) to P_H 6.6. While there was not much difference between the fermentation when 10 grams and 5 grams of alcohol were used per liter, a larger number of cultures fermented the alcohol in these cases than when only 3 grams was used. In general, when a culture fermented ethyl it fermented propyl alcohol, but this was not true in all cases. Usually when amyl alcohol was fermented ethyl and propyl were fermented also, but there were several exceptions. Only one of the cultures which fermented mannite also fermented the monoatomic alcohols. Cultures 121, 133, and 134, which fermented glycerin, were not able to ferment any of the monoatomic alcohols.

TABLE 9.—*Fermentation of the alcohols.*

Culture No.	Ethyl, grams per liter.			Propyl, grams per liter.			Amyl, grams per liter.			Mannite.	Glycerin.
	10	5	3	10	5	3	10	5	3		
10	−	−	−	−	−	−	−	−	−	−	−
12	−	−	−	−	−	−	−	−	−	−	−
31	−	+	+	−	+	+	+	−	−	+	−
49	−	+	+	−	+	+	+	−	−	−	−
59	+	+	−	+	+	+	−	−	+	−	−
60	+	+	+	+	+	+	+	+	+	−	−
61	+	+	−	+	+	+	+	−	−	−	−
62	−	−	−	+	+	+	+	−	−	−	−
66	+	+	−	+	+	+	+	+	+	−	−
72	−	−	−	−	−	−	−	−	−	+	−
73	+	+	−	−	−	−	−	−	−	−	−
101	+	+	−	+	+	+	−	−	−	−	−
102	+	+	−	+	+	+	−	−	−	−	−
103	−	−	−	−	−	−	−	−	−	−	−
104	−	−	−	+	+	+	−	−	−	−	−
105	−	−	+	+	−	−	−	−	−	−	−
106	+	+	−	+	+	+	+	+	+	−	−
107	−	+	−	−	+	+	−	−	−	−	−
108	+	+	−	+	+	+	−	−	−	−	−
111	+	+	+	+	+	+	+	+	+	−	−
112	−	+	−	−	−	−	−	−	−	−	−
113	−	+	−	+	+	−	−	−	+	−	−
114	+	+	−	+	+	+	−	−	−	−	−
116	−	+	−	+	+	+	−	−	−	−	−
117	+	+	−	+	+	+	−	−	−	−	−
118	+	+	+	+	+	+	+	−	+	−	−
119	−	+	−	+	+	+	+	−	−	−	−
120	−	−	−	−	−	−	−	−	−	−	−
121	−	−	−	−	−	−	−	−	−	+	+
122	+	+	−	+	+	+	+	+	+	−	−

TABLE 9.—*Fermentation of the alcohols*—Continued.

Culture No.	Ethyl, grams per liter.			Propyl, grams per liter.			Amyl, grams per liter.			Mannite.	Glycerin.
	10	5	3	10	5	3	10	5	3		
123	−	−	−	−	−	−	−	−	−	−	−
124	−	+	−	+	+	+	−	−	−	−	−
125	+	+	+	+	+	+	−	−	−	−	−
126	+	+	+	+	+	+	−	−	−	−	−
128	+	+	−	+	+	+	+	−	−	−	−
130	+	+	−	+	+	+	+	+	−	−	−
131	−	−	−	+	+	+	−	−	−	−	−
132	+	+	−	+	+	+	−	−	−	−	−
133	−	−	−	−	−	−	−	−	−	+	+
134	−	−	−	−	−	−	−	−	−	+	+
135	−	+	−	+	+	+	−	−	−	−	−
136	+	−	−	+	+	+	−	−	−	−	−
137	−	−	−	−	+	+	−	−	−	−	−
138	+	+	−	+	+	+	−	−	−	−	−
139	+	+	−	+	+	+	+	+	+	−	−
140	−	+	−	+	+	+	−	−	−	−	−
141	−	−	−	−	−	−	−	−	−	−	−
142	+	+	−	+	+	+	+	−	+	−	−
143	−	+	−	−	+	+	+	−	−	−	−
144	−	+	−	+	+	+	−	−	−	−	−
145	+	+	−	+	+	+	+	+	+	−	−
146	+	+	−	+	+	+	+	−	−	−	−
147	+	+	−	+	+	+	+	+	+	−	−
303	+	+	−	+	+	−	−	−	−	−	−
309	−	−	−	−	−	−	−	−	−	−	−
314	−	−	−	−	−	−	−	−	−	−	−
317	+	+	−	+	+	+	−	−	−	−	−
319	+	+	+	−	−	−	−	−	−	−	−
321	+	+	+	+	+	−	+	−	+	−	−
322	+	+	+	−	+	−	+	−	+	−	−
325	+	+	+	+	−	−	+	−	−	−	−
326	+	+	+	−	+	+	+	−	+	−	−
340	+	+	−	+	+	−	−	−	−	−	−
353	−	−	−	−	−	−	−	−	−	−	−
354	+	+	−	+	+	+	−	−	−	−	−
356	+	+	+	+	+	+	−	−	−	−	−
361	+	+	+	−	−	−	+	−	+	−	−
362	−	−	−	−	−	−	−	−	−	−	−
Total positive	37	48	15	43	48	43	24	9	16	5	3

NOTE.—No fermentation in the following alcohols—methyl, dulcite, adonite.

It is evident from these results that the polyatomic alcohols adonite, dulcite, mannite, and glycerin are satisfactory sources of carbon for the alkali-forming group of bacteria and were of no particular value in the study of the bacteria under discussion in this paper. On the other hand the monoatomic alcohols ethyl, propyl, and amyl were fermented by a larger number of cultures, and while the fermentations were relatively slight it is believed that they may be of some value in further studies of the alkali-forming bacteria.

FERMENTATION OF SALTS OF ORGANIC ACIDS.

It has been shown previously in this paper that the alkaline reaction in milk produced by the alkali-forming organisms was due to the oxidation of the salts of citric acid. Since these bacteria can ferment the salts of citric acid it seemed quite probable that other organic-

acid salts might be utilized as a source of carbon and thereby become valuable substances for fermentation tests.

In 1895 Maassen (7) studied the fermentation of 21 different organic-acid salts by 52 varieties of bacteria. He obtained some growth with all these cultures in media containing the various organic-acid salts. As Maassen used these salts in a medium containing peptone, it is not surprising that he obtained positive results with all the organic acids, for the reason that the carbon could probably have been obtained by the bacteria from either the organic acid or the peptone, according to which one was the more available. If these organic-acid salts are to serve as carbon-containing test substances they must be used in a medium containing no other source of carbon.

In order to determine the value of the salts of the organic acids as a source of carbon, the fermentation of 24 of these salts by the alkali-forming group of bacteria was studied. The fermentation was determined in a synthetic medium of the following composition:

$NaNH_4HPO$	gram..	0.50
NaH_2PO_4	do....	.17
KCl	do....	.20
Water, distilled	c. c..	1,000
Acid (neutralized with NaOH)	grams..	1.2

Urea and hippuric and uric acids were also used in the following media:

Urea	grams..	2.00	Hippuric acid	gram.	1.0	Uric acid	gram..	1.0
Na_2HPO_4	gram..	.50	NaOH quantity sufficient to neutralize the acid	gram.	.50	NaOH	grams..	8.00
KCl	do....	.10				KCl	gram..	.10
NaH_2PO_4	do....	.17	NaH_2PO_4	do..	.17	Water, distilled	c.c..	1,000
Water, distilled (filter through Berkefeld)	c.c..	1,000	KCl	do..	.10			
			Water	c. c..	1,000			

It is evident from the composition of the medium that if growth occurs the carbon must be obtained from an organic-acid salt. The fermentation of the organic-acid salts was measured by the change in the hydrogen-ion concentration. A change of more than P_H 0.2 being recorded as a positive fermentation. An exception is taken in recording the results of the fermentation of butyric, valeric, and caproic acids where any change from the control is recorded as positive because of the possibility that these acids were split into simpler acids, and these simpler acids in turn oxidized to alkaline carbonates. The final hydrogen-ion concentration of the medium would in such cases depend upon the point in the fermentation at which it was determined. In every case a change in hydrogen-ion concentration from the control showed growth in the tubes. The complete result of the study is given in Table 10. It will be seen in the tabulated

results in the table that nearly all the organic-acid salts used were fermented to a greater or less extent. In the table are the results of hippuric and uric acids and urea, these substances being included merely for the sake of convenience; their fermentations will be discussed later.

Before proceeding with the fermentation of the salts of the organic acids it is necessary to call attention to the fact that the growth in the synthetic medium as used in this investigation with the organic-acid salts as a source of carbon was usually quite different in appearance from the growth obtained in the sugar broths. It was found that the alkali-forming bacteria frequently grew in a mass in the bottom of the tube and left the medium perfectly clear. Often when the greatest change in the hydrogen-ion concentration was noticed there was little appearance of growth in the tubes; at other times the medium became extremely cloudy.

In connection with the fermentation of these organic-acid salts an attempt has been made to determine from which radical or radicals the carbon was obtained. The organic acids are grouped, therefore, in Table 11 according to the radicals they contain. For example citric, malic, and lactic acids are grouped together for discussion because they contain a secondary or tertiary alcohol radical linked to a carboxyl and to a methyl radical.

It will be seen in Table 11 that all the 68 cultures studied were able to utilize carbon from the sodium salt of pyruvic acid. The table gives the structural formula of the different acids but it must be remembered that the sodium salt was employed. Pyruvic acid ($CH_3COCOOH$) comprises a ketone (CO) radical connecting a methyl (CH_3) and carboxyl (COOH). It seems that carbon was readily available from an acid of this structure. This acid was sterilized by passage through a Berkefeld filter so as to prevent decomposition by heat. The cultures also obtained their carbon readily from other oxyacids, such as citric, malic, and lactic. In such acids an alcohol radical connects the carboxyl to the methyl group, as illustrated by

$$\begin{array}{l} CH_3 \\ | \\ CHOH \\ | \\ COOH \end{array}$$

lactic acid. The cultures were able to obtain their carbon almost as well from salts of such acids as succinic, acetic, propionic, butyric, valeric, and caproic, in which there is no alcohol radical, but one methyl group is linked to a carboxyl.

TABLE 10.—*Complete results of the fermentations of the organic-acid salts and urea in terms of* P_H *values.*

Culture No.	Citric.	Malic.	Lactic.	Succinic.	Acetic.	Propionic.	Butyric.	Isobutyric.	α Oxybutyric.	β Oxybutyric.	Valeric.	Isovaleric.	Caproic.	Mucic.	Glyceric.	Tartaric.	Malonic.	Formic.	Benzoic.	Salicylic.	Hippuric.	Uric.	Urea.
Control	7.2	6.9	6.9	7.1	6.9	6.9	6.9	6.9	6.6	6.9	7.0	6.9	6.9	6.9	6.9	7.0	7.0	6.9	6.9	6.8	6.9	7.4	7.0
119	8.3	8.9	7.4	8.5	7.5	7.2	6.7	7.2	6.8	7.6	6.4	7.1	6.6	6.9	6.9	7.0	7.0	7.4	6.9	6.8	6.9	7.4	7.4
130	8.8	8.9	7.7	8.5	8.1	7.6	7.2	7.2	7.0	6.9	6.2	7.2	6.3	6.9	6.9	7.0	7.0	7.7	7.2	7.2	6.9	7.4	7.5
131	8.3	8.7	7.7	8.5	8.0	7.5	7.0	7.5	7.0	7.6	6.5	7.1	6.8	6.9	6.9	7.0	7.0	7.6	7.1	6.8	6.9	7.4	8.5
138	8.5	8.9	7.7	8.5	7.5	7.9	6.7	7.3	7.1	7.5	6.4	7.1	6.3	6.9	6.9	7.0	7.0	7.4	7.1	6.8	6.9	7.4	7.0
140	8.3	8.9	7.7	8.2	7.9	8.0	6.5	7.3	7.1	7.6	6.4	7.2	6.3	6.9	6.9	7.0	7.0	7.3	7.1	6.8	6.9	7.4	7.0
354	8.2	8.8	7.7	8.8	7.4	6.9	6.5	7.3	7.1	7.5	6.3	7.2	6.8	6.9	6.9	7.0	7.0	7.8	6.9	6.8	6.9	7.4	8.3
10	8.8	8.8	7.9	8.5	8.1	7.7	7.8	7.5	7.0	7.8	7.2	7.3	7.5	7.6	8.2	7.0	7.0	8.5	(1)	6.8	(1)	8.6	7.0
12	8.8	8.8	7.9	8.5	8.4	7.7	7.8	7.5	7.1	7.8	7.6	7.4	7.6	7.6	8.2	7.0	7.0	8.5	(1)	6.8	(1)	8.6	7.0
31	8.8	8.9	8.2	8.8	8.6	8.0	7.5	7.4	7.2	7.8	7.1	7.4	7.2	6.9	6.9	7.0	7.0	8.2	7.1	6.8	7.5	7.4	8.7
49	8.7	8.8	8.0	8.4	8.3	7.5	7.8	7.5	7.0	7.8	7.2	7.4	7.6	7.5	8.2	7.0	7.0	8.5	7.3	6.8	(1)	8.6	7.0
59	8.7	8.9	7.6	8.5	8.2	7.8	7.7	7.5	7.1	7.6	6.9	7.2	7.4	6.9	6.9	7.0	8.7	6.9	6.9	6.8	6.9	7.4	8.2
60	8.8	8.9	8.1	8.7	8.4	8.0	7.6	7.4	7.5	7.3	6.5	7.2	7.2	6.9	6.9	7.0	8.7	6.9	7.3	7.3	6.9	7.4	8.1
61	8.6	8.8	7.7	8.3	8.2	7.8	7.7	7.4	7.1	7.6	6.9	7.2	7.4	6.9	6.9	7.0	8.7	6.9	6.9	6.8	6.9	7.4	8.4
62	8.7	8.9	8.1	8.5	8.4	8.0	7.6	7.3	7.2	7.6	6.5	7.2	7.2	6.9	6.9	7.0	8.7	6.9	7.3	7.2	6.9	7.4	8.3
66	8.7	8.8	8.0	8.6	8.2	7.9	7.6	7.5	7.4	7.6	6.5	7.2	6.5	6.9	6.9	7.0	7.0	6.9	7.2	7.2	7.3	7.4	8.3
72	8.8	8.9	8.2	8.7	8.5	8.0	7.6	7.6	7.5	7.8	7.3	7.4	7.1	6.9	6.9	7.0	7.0	8.5	7.1	6.8	7.6	7.4	8.7
73	7.2	7.7	7.7	7.1	7.4	6.9	6.9	6.9	6.6	7.3	7.0	6.9	6.9	6.9	6.9	7.0	7.0	6.9	6.9	6.8	6.9	7.4	7.9
101	8.3	8.8	7.8	8.1	7.6	7.4	6.4	7.3	7.2	7.5	6.2	7.2	6.2	6.9	6.9	7.0	7.0	7.5	7.1	6.8	6.9	7.4	7.0
102	8.3	8.9	7.8	8.2	7.4	8.0	6.2	7.3	7.1	7.3	6.2	7.2	6.2	6.9	6.9	7.0	7.0	7.5	7.1	6.8	6.9	7.4	7.0
103	7.2	7.2	7.3	7.1	6.9	6.9	6.9	6.9	6.6	7.2	7.0	6.9	6.9	6.9	6.9	7.0	7.0	6.9	6.9	6.8	6.9	7.4	7.0
104	8.5	8.9	7.6	8.1	7.5	7.4	6.4	7.3	6.9	7.6	6.2	7.2	6.5	6.9	6.9	7.0	7.0	7.2	7.1	6.8	6.9	7.4	7.0
105	8.5	8.9	7.6	8.2	7.7	7.1	6.3	7.3	6.9	7.2	6.3	7.1	6.5	6.9	6.9	7.0	7.0	7.2	7.1	6.8	6.9	7.4	7.0
106	8.9	8.9	8.1	8.8	8.4	8.0	7.6	7.5	7.5	7.4	6.9	7.2	7.1	6.9	6.9	7.0	7.0	8.5	7.3	7.2	6.9	7.4	8.5
107	8.7	8.8	8.1	8.1	8.5	8.0	7.8	7.5	7.2	7.7	7.2	7.4	7.4	7.6	8.5	7.0	7.0	8.6	7.3	6.8	8.0	7.4	7.0
108	8.7	8.9	7.6	8.2	7.7	7.5	6.2	7.3	7.0	6.9	6.2	7.2	6.3	6.9	6.9	7.0	7.0	7.3	7.1	6.8	6.9	7.4	7.0
111	8.8	8.9	7.6	8.5	8.3	8.0	7.3	7.6	7.4	7.0	6.2	7.2	6.3	6.9	6.9	7.0	7.0	8.1	7.3	7.3	6.9	7.4	7.8
112	8.5	8.9	8.0	8.3	8.2	7.8	7.8	7.4	7.0	7.6	7.3	7.4	7.6	7.5	8.3	8.5	7.0	8.6	6.9	6.8	7.9	8.6	7.0
113	8.8	8.4	7.7	8.3	8.4	8.0	7.8	7.5	7.1	7.7	7.4	7.4	7.6	7.6	8.4	7.0	7.0	8.6	(1)	6.8	(1)	7.4	7.0
114	8.2	8.7	7.6	7.9	7.3	7.3	6.4	7.3	6.6	6.9	6.2	7.2	6.2	6.9	6.9	8.4	7.0	6.9	6.9	6.8	6.9	7.4	7.0
116	8.7	8.9	7.5	8.2	7.5	7.3	7.1	7.3	6.9	7.6	6.2	7.1	6.5	6.9	6.9	7.0	7.0	7.5	7.1	6.8	6.9	7.4	7.0
117	8.5	8.9	7.6	8.2	7.6	7.3	6.3	7.3	6.9	7.8	6.2	7.2	6.3	6.9	6.9	7.0	7.0	7.5	7.1	6.8	6.9	7.4	7.0
118	8.8	8.9	7.7	8.6	8.2	8.1	7.6	7.7	7.3	7.3	6.5	7.4	6.5	6.9	6.9	7.0	7.0	8.5	7.3	7.2	6.9	7.4	7.8
120	8.4	8.9	8.0	8.3	8.2	7.8	7.7	7.3	7.3	7.7	7.4	7.2	7.6	7.5	8.2	8.0	7.0	8.7	6.9	6.8	8.7	8.2	8.5
121	8.0	8.3	8.0	8.4	7.8	8.9	6.9	6.9	6.6	6.9	7.0	6.9	6.9	6.9	7.9	7.0	8.6	7.3	6.9	6.8	7.5	7.4	7.4
122	8.8	8.9	7.9	8.7	8.4	8.0	7.6	7.5	7.3	7.6	6.5	7.3	6.3	6.9	6.9	7.0	7.0	6.9	7.3	7.2	6.9	7.4	8.4
123	8.9	8.7	7.7	8.5	8.5	7.7	7.8	7.5	7.1	7.6	7.4	7.3	7.5	7.5	8.2	7.0	7.0	8.5	6.9	6.8	8.0	8.5	7.0
124	8.2	8.8	7.6	8.1	7.4	7.3	6.7	7.3	6.8	6.9	6.5	6.9	6.6	6.9	6.9	7.0	7.0	7.3	6.9	6.8	6.9	7.4	7.5

125	8.8	8.8	7.6	8.2	7.7	7.4	6.7	7.3	7.1	6.9	6.2	7.1	6.3	6.9	6.9	7.0	7.0	7.5	6.9	6.8	6.9	7.4	7.9
126	8.6	8.9	7.6	8.0	7.7	7.3	6.5	7.3	6.9	7.1	6.6	7.0	6.8	6.9	6.9	7.0	7.0	7.4	7.1	6.8	6.9	7.4	7.0
128	8.2	8.8	7.4	7.8	7.4	7.3	6.4	7.2	6.6	6.9	6.2	7.1	6.3	6.9	6.9	8.2	7.0	6.9	6.9	6.8	6.9	7.4	7.0
132	8.8	8.8	7.6	8.2	7.8	7.2	7.3	7.3	7.1	7.6	6.5	7.1	6.8	6.9	6.9	8.5	7.0	7.6	7.1	6.8	6.9	7.4	7.0
133	8.8	8.4	8.0	8.2	8.4	6.9	6.9	6.9	6.6	6.9	7.0	6.9	6.9	6.9	8.2	7.0	8.5	8.5	6.9	6.8	7.3	7.4	6.5
134	8.1	8.4	7.6	8.2	8.1	6.9	6.9	6.9	6.6	6.9	7.0	6.9	6.9	6.9	7.8	7.0	8.5	8.1	6.9	6.8	7.3	7.4	8.5
135	8.6	8.8	7.8	8.1	7.5	7.8	7.0	7.3	7.1	7.7	6.2	7.1	6.5	6.9	6.9	7.0	7.0	7.4	7.2	6.8	6.9	7.4	8.6
136	8.7	8.8	7.8	8.0	7.7	7.4	6.6	7.2	7.1	7.4	6.2	7.1	6.5	6.9	6.9	7.0	7.0	7.4	6.9	6.8	6.9	7.4	7.0
137	8.8	8.8	8.1	8.3	8.2	7.9	7.8	7.6	7.1	7.6	7.7	7.3	7.4	7.6	8.3	7.0	7.0	8.6	7.3	6.8	7.7	7.4	7.0
139	8.8	9.0	8.0	8.5	8.2	8.0	6.7	7.4	7.3	7.6	6.5	7.3	6.9	6.9	6.9	7.0	7.0	7.4	7.3	7.3	6.9	7.4	8.3
141	8.5	8.9	8.0	8.2	7.7	7.9	7.5	7.3	7.4	7.7	7.3	7.2	7.5	7.5	8.2	8.3	7.0	8.7	6.9	6.8	7.7	8.2	7.5
142	8.7	8.8	8.1	8.9	8.5	7.8	7.5	7.4	7.1	7.2	6.6	7.2	7.2	6.9	6.9	7.0	8.8	6.9	7.2	7.3	6.9	7.4	7.8
143	8.8	8.9	7.8	8.5	8.5	7.6	7.8	7.5	7.0	7.7	7.6	7.3	7.2	7.5	8.2	7.0	7.0	8.5	6.9	6.8	8.0	8.7	7.0
144	8.8	8.8	7.4	8.4	8.0	7.3	7.0	7.3	6.6	7.3	6.6	6.9	6.5	6.9	6.9	7.0	7.0	7.6	6.9	6.8	6.9	7.4	8.2
145	8.5	8.9	8.0	8.8	8.4	8.1	7.6	7.5	7.4	7.6	6.5	7.2	7.2	6.9	6.9	7.0	8.9	6.9	7.4	7.3	6.9	7.4	8.1
146	8.5	8.9	7.6	8.2	7.8	7.5	6.5	7.3	7.1	7.3	6.2	7.2	6.2	6.9	6.9	7.0	7.0	7.5	7.1	6.8	6.9	7.4	7.0
147	8.8	9.0	8.1	8.7	8.3	7.8	7.6	7.3	7.3	7.5	6.5	7.2	7.1	6.9	6.9	7.0	7.0	6.9	7.3	7.2	6.9	7.4	8.5
303	7.2	7.3	7.3	7.4	7.5	6.9	7.1	7.3	6.6	7.5	7.0	7.2	6.9	6.9	6.9	7.0	7.0	6.9	6.9	6.8	6.9	7.4	7.7
309	7.2	6.9	6.9	7.1	7.3	7.2	7.1	7.1	6.6	7.3	7.0	6.9	6.9	6.9	6.9	7.0	7.0	6.9	6.9	6.8	6.9	7.4	8.3
314	8.8	7.9	7.4	7.7	8.0	7.3	7.4	7.3	6.6	7.6	7.2	6.9	7.2	6.9	7.9	7.0	8.7	8.5	6.9	6.8	6.9	7.4	7.8
317	8.2	8.7	7.6	8.0	7.3	7.2	6.4	7.2	6.6	6.9	6.2	7.1	6.2	6.9	6.9	8.6	7.0	6.9	6.9	6.8	6.9	7.4	7.0
319	8.5	6.9	8.1	7.1	8.2	7.9	7.9	7.3	7.2	7.2	7.4	7.1	7.2	6.9	6.9	7.0	7.0	8.5	6.9	6.8	6.9	7.4	8.7
321	8.5	7.4	7.9	8.5	8.2	7.9	7.6	7.3	7.0	7.1	7.3	7.2	7.4	6.9	6.9	7.0	7.0	8.0	6.9	6.8	6.9	7.4	8.7
322	8.5	7.6	7.6	8.6	8.2	8.0	7.7	7.3	7.2	7.2	7.4	7.2	7.2	6.9	6.9	7.0	7.0	8.5	6.9	6.8	6.9	7.4	8.8
325	8.5	7.6	8.1	8.1	7.4	8.0	7.6	7.3	7.1	7.1	7.1	7.2	7.3	6.9	6.9	7.0	7.0	7.9	6.9	6.8	6.9	7.4	8.8
326	8.5	7.4	7.5	8.0	7.9	7.9	7.7	7.3	6.9	6.9	7.2	7.2	7.4	6.9	6.9	7.0	7.0	8.2	6.9	6.8	6.9	7.4	8.8
340	7.9	8.0	7.3	8.6	7.4	7.1	6.8	7.1	6.6	7.3	6.8	6.9	6.6	6.9	6.9	7.0	7.0	6.9	6.9	6.8	6.9	7.4	7.6
353	7.2	8.2	7.8	8.5	8.1	7.3	7.2	7.2	7.1	7.7	7.4	7.2	7.4	6.9	7.9	7.0	7.0	8.5	6.9	6.8	6.9	7.7	7.0
356	8.7	7.2	7.8	8.1	8.2	7.1	7.5	7.3	6.6	7.1	7.3	6.9	7.4	6.9	6.9	7.0	8.7	8.5	6.9	6.8	6.9	7.4	8.8
361	8.3	8.0	8.1	8.1	8.2	7.7	7.7	7.3	7.1	7.2	7.4	7.2	7.2	6.9	6.9	7.0	7.0	8.1	6.9	6.8	6.9	7.4	8.8
362	8.2	7.1	7.1	7.7	7.5	6.9	7.2	6.9	7.0	7.1	7.0	6.9	6.9	6.9	6.9	7.0	7.0	8.2	6.9	6.8	6.9	7.4	8.5
Number positive	63	65	67	64	67	58	51	60	52	52	49	43	52	11	16	7	11	51	16	12	13	8	41

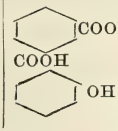
NOTE.—All cultures fermented pyruvic acid and none fermented oxalic and glycolic acids.

[1] Reactions were not determined on account of high color of the medium caused by bacterial growth.

The table shows that only a few cultures were able to ferment mucic, tartaric, and glyceric acid, in which there is a secondary alcohol radical connecting a carboxyl with another alcohol radical, as in mucic $COOH(CHOH)_4COOH$, tartaric $COOH(CHOH)_2COOH$, and glyceric $CH_2OH(CHOH)COOH$. It seems to be somewhat easier for this group of bacteria to obtain carbon from glyceric acid when a primary alcohol radical (CH_2OH) replaced a secondary (CHOH). Only 7 cultures were able to ferment tartaric acid, while none of the 68 cultures were able to utilize glycolic acid. Tartaric acid has a structural formula $\begin{matrix} CHOH\ COOH \\ | \\ CHOH\ COOH \end{matrix}$ where an alcohol radical is linked to a carboxyl and another alcohol, while in glycolic $\begin{matrix} CH_2OH \\ | \\ COOH \end{matrix}$ an alcohol radical is merely linked to a carboxyl. This difference in manner in which the alcohol radical is linked to the carboxyl may make it possible for some bacteria to ferment tartaric acid. In malonic acid $CH_2\begin{matrix} /COOH \\ \backslash COOH \end{matrix}$ two carboxyls are linked by a methyl group and it was found that 11 of the 68 cultures were able to obtain carbon from this source, while none of them were able to ferment oxalic acid $\begin{matrix} COOH \\ | \\ COOH \end{matrix}$ when 2 carboxyl groups are joined directly. In this case it seems that the presence of the methyl group connecting the carboxyl group makes it possible for a few cultures to obtain carbon, probably from the methyl radical. Our results indicate that carbon is not obtained from the carboxyl group, since none of the cultures were able to utilize oxalic acid. This, however, may be due to the poisonous character of the acid. Whether this nonfermentability of the exalate is due to the configuration of the molecule or to the poisonous character of the compound, it is interesting to note that the work of Doryland (5) confirms this result as he was unable to obtain fermentation of oxalate when used as the only source of carbon in a synthetic medium in which 225 different bacteria were studied.

A large number of the cultures could utilize carbon from the sodium salt of formic acid. It is possible to consider the structure of this acid $HO–C\begin{matrix} //O \\ \backslash H \end{matrix}$ and it seems evident that the carbon can be obtained from the aldehyd radical. If the structure is taken as H–COOH the organism would have to obtain carbon from a carboxyl group, and if they are able to do this with formic acid they should be expected to ferment oxalic acid.

TABLE 11.—*Summary of the fermentation of the organic-acid salts.*

Acid.	Chemical structure.	Number of cultures fermenting.	Remarks.
Pyruvic	$CH_3\,CO\,COOH$	68	Ketone connecting a carboxyl and a methyl radical.
Citric	$\begin{matrix}CH_2-COOH\\COH-COOH\\CH_2-COOH\end{matrix}$	63	Secondary or tertiary alcohol radical linked to a carboxyl and to a methyl radical.
Malic	$\begin{matrix}CH-(OH)\,COOH\\CH_2-COOH\end{matrix}$	65	
Lactic	$\begin{matrix}CH_3\\CHOH\\COOH\end{matrix}$	67	
Succinic	$\begin{matrix}CH_2-COOH\\CH_2-COOH\end{matrix}$	64	No alcohol radical. Methyl linked to a carboxyl radical.
Acetic	$CH_3\,COOH$	67	
Propionic	$CH_3\,CH_2\,COOH$	58	
Butyric	$CH_3\,CH_2\,CH_2\,COOH$	51	
Valeric	$CH_3\,CH_2\,CH_2\,CH_2\,COOH$	49	
Caproic	$CH_3\,CH_2\,CH_2\,CH_2\,CH_2\,COOH$	52	
Mucic	$COOH\,(CHOH)\,(CHOH)\,(CHOH)\,(CHOH)\,COOH$.	11	Alcohol radical linked to a carboxyl and to another alcohol radical.
Glyceric	$CH_2OH\,(CHOH)\,COOH$	16	
Tartaric	$COOH\,(CHOH)\,(CHOH)\,COOH$	7	
Oxalic	$\begin{matrix}COOH\\COOH\end{matrix}$	0	Two carboxyls linked together.
Malonic	$CH_2\begin{matrix}COOH\\COOH\end{matrix}$	11	Two carboxyls linked with methyl radical.
Glycolic	$\begin{matrix}CH_2OH\\COOH\end{matrix}$	0	Primary alcohol linked with a carboxyl.
Formic	$H-C\begin{matrix}O\\OH\end{matrix}$	51	Aldehyd radical linked to hydroxyl.
Benzoic		16	Benzol ring.
Salicylic		12	

Total number of cultures, 68.

Some of the bacteria were able to obtain their carbon from benzoic and salicylic acids, in which case it was apparently necessary for the bacteria to split the benzol ring. If they could obtain their carbon without splitting the benzol ring they would have to take it from the COOH group, and if it was possible in this case, it would be expected that they could also obtain carbon from the COOH group of oxalic acid. The sodium salt of benzoic acid was fermented by more cultures than that of salicylic acid, as is shown by the fact that 16 cultures fermented benzoic and only 12 salicylic. The stronger antiseptic property of salicylic acid, which possibly is due to the presence of the hydroxyl group, may make it difficult for some of the bacteria to grow in the presence of the acid salts.

The results of the study of the fermentation of the organic-acid salts indicate that some of the bacteria could obtain their carbon from the methyl, alcohol, or aldehyd (as in formic acid) radical of the salts of organic acids but not from the carboxyl group. Carbon was most easily obtained from the methyl group, as is shown from Table 11, where it is seen that most of the cultures were able to obtain their carbon from acids which had no alcohol radical, such as succinic or acetic. They were able to obtain it to about the same extent from citric, malic, and lactic acids, which contained both the methyl and alcohol radicals. In this case it is believed that the methyl radical supplies the carbon, since acids containing merely the alcohol radicals were not readily fermented. Only a small number of the cultures utilized the carbon from the alcohol radical where it was linked with a carboxyl and another alcohol radical, as in mucic, tartaric, and glyceric acid. The carbon did not seem to be utilized from an alcohol radical linked only to a carboxyl radical, as in glycolic acid. Pyruvic acid was easily fermented by all the cultures, and in this case the carbon may come from either the methyl or ketone radical. A. Fischer mentions in his book "The Structure and Functions of Bacteria," published in 1897, that bacteria are able to utilize carbon from the methyl radical more readily than from the alcohol radicals and they are unable to utilize the carbon from the carboxyl radical, as in oxalic acid. Our results confirm his opinion in the matter.

When the salts of organic acids were fermented an alkaline reaction was produced in most cases, the exceptions being butyric, valeric, and caproic acids. In the case of these acids the fermentation may proceed in one of two ways: Either the methyl radicals may be oxidized until a carbonate is produced and the medium becomes alkaline, or the acid may be split into other acids containing fewer carbon atoms. For example butyric may be oxidized as follows:

$$\underset{\text{butyric}}{CH_3\,CH_2\,CH_2\,COOH} + O_2 = 2\,\underset{\text{acetic}}{CH_3\,COOH}$$

$$\underset{\text{butyric}}{CH_3\,CH_2\,CH_2\,COOH} + O_2 = \underset{\text{formic}}{HCOOH} + \underset{\text{propionic}}{CH_3\,CH_2\,COOH}$$

The oxidation of butyric acid to other acids may account for the increased hydrogen-ion concentration produced by certain alkali bacteria, since under ordinary conditions this concentration is lowered by the production of carbonates. Both these forms of fermentation may take place at the same time, but in the great majority of cases one or the other predominated, as is shown in Table 10. While many of the cultures produced an acid reaction in n-butyric and n-valeric acids, all those which fermented isobutyric, α or β oxybutyric, and isovaleric acids produced an alkaline reaction.

In order to show whether the alkaline reaction was due to the production of carbonates or bicarbonates, or both, Table 12 has been prepared, using the organic acids which were utilized as test substances in this investigation. Here again attention is called to the fact that the essential process in the production of an alkaline reaction is the replacement of the relatively strong organic acids by the relatively weak carbonic acid. The figures in the table are based on the complete oxidation to the carbonate or bicarbonate of 100 c. c. of a 0.1 per cent solution of the acid. In the next to the last column of the table the tenth-normal equivalent is given in cubic centimeters for both the carbonate and bicarbonate. This table should be studied in connection with the plot in figure 1 which represents the change of any of the test acid salt media from the initial P_H 6.9. The resulting hydrogen-ion concentration obtained when known amounts of tenth-normal $NaHCO_3$ were added to the medium above is represented by one curve. The other curve was obtained similarly by plotting the resulting hydrogen-ion concentration when known amounts of tenth-normal Na_2CO_3 were added to the same synthetic medium. It will be noted that the large quantity of alkali generated by some of the fermentations could not possibly be due to the complete oxidation of the acid to bicarbonate. Let us take, for example, propionic acid; on complete oxidation this acid yields an equivalent of 1.3 cubic centimeters of tenth-normal alkali. From the curve it is seen that 1.3 cubic centimeters of tenth-normal alkali gives a hydrogen-ion concentration of P_H 7.9 while the average hydrogen-ion concentration of the alkali group of bacteria which fermented propionic acid was P_H 7.4.

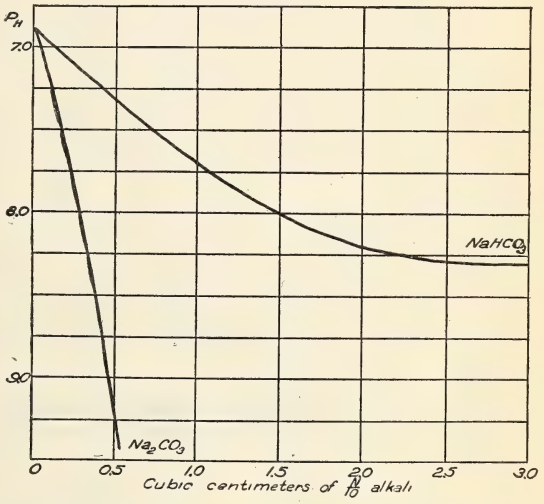


FIG. 1.—Curve showing effect of addition of bicarbonate and sodium carbonate on the reaction of the sodium-ammonium-phosphate medium.

From this it is evident that the entire amount of alkali produced from this organic-acid salt might be accounted for by the production of sodium bicarbonate. On the contrary, if we examine the figures for glyceric acid it will be found that this acid on complete oxidation yields an equivalent of 0.94 cubic centimeters tenth-normal alkali, which corre-

sponds on the plot to a hydrogen-ion concentration of P_H 7.65. The average hydrogen-ion of the cultures which fermented this acid was P_H 8.1; therefore the alkali produced by the fermentation of this organic-acid salt can not be due to the formation of sodium bicarbonate alone but must be due to sodium carbonate, or a combination of the two. In the 14 acids calculated and shown in Table 12 by similar manipulation of the plot, it was found that 6 of the acid salts, namely, citric, malic, succinic, glyceric, tartaric, and malonic produced alkali enough to account for the production of sodium carbonate alone, or the carbonate and bicarbonate together. The remaining acid salts can furnish alkali enough upon complete oxidation to account for the entire alkalinity either through the formation of sodium bicarbonate alone or a combination of the two. These plots do not show exactly the conditions found in the cultures when there is a replacement of a strong acid radical by the weak carbonic acid. Although these plots show only the effect of the addition of carbonates, they nevertheless clearly indicate the tendency of both the carbonate and bicarbonate to decrease the P_H value of the medium. They further show the relatively weak effect of bicarbonate when the medium is at a P_H included in the zone where the carbonic-bicarbonate equilibrium is obtained, and indicate very nearly the quantitative effect of the replacement mentioned above provided the acid replaced has a dissociation constant much larger than that of carbonic acid. This last condition does in fact apply to all the acids studied.

TABLE 12.—*Theoretical carbonate production from organic-acid salts, together with the average P_H value produced by the alkali-forming bacteria.*

Acid.	Na_2CO_3 obtained by complete oxidation of 0.01 gram of acid.	$NaHCO_3$ obtained by complete oxidation of 0.01 gram of acid.	Equivalent in N/10 alkali.	Average hydrogen-ion concentration.
	Gram.	*Gram.*	*C. c.*	P_H.
Citric	0.008275	0.01311	1.562	8.53
Malic	.007905	.01252	1.490	8.56
Lactic	.005887	.009333	1.110	7.72
Succinic	.008974	.01422	1.693	8.32
Acetic	.008822	.01399	1.665	8.00
Proprionic	.007158	.01134	1.351	7.42
Butyric	.006019	.09541	1.136	7.27
Mucic	.005165	.007997	.9520	7.53
Glyceric	.004996	.007921	.9429	8.10
Tartaric	.007062	.01119	1.333	8.33
Malonic	.01020	.01615	1.923	8.66
Formic	.0152	.01826	2.169	8.20
Benzoic	.004341	.006882	.8191	7.15
Salicylic	.003838	.06084	.7241	7.25

When hippuric and uric acids and urea were used no other source of nitrogen was supplied; therefore the organisms had to obtain both their necessary carbon and nitrogen requirements from these com-

pounds. The urea was sterilized through a Berkefeld filter, so there was no ammonium carbonate present as would have been the case if the ordinary method of steam sterilization had been employed. Ammonia was an end product from all three of these test substances, and with urea the only alkali end product possible. Reference to Table 10 shows that 13 of the cultures fermented hippuric acid, 8 uric acid, and 41 urea. In addition to the 68 cultures of alkali-forming bacteria isolated from dairy products, the fermentation of the organic-acid salts was tried with 55 known cultures, namely, 37 strains of dysentery, 6 of proteus, 6 of typhoid, 3 of paratyphoid, and 1 each of *B. abortus*, *B. bronchisepticus*, *B. fæcalis alkaligenes*, and *B. enteriditis*. The results of the fermentation of the citric and lactic salts and litmus-milk reaction are given in Table 13. It is evident from the results that all the cultures fermented the citrate and a large number of them the lactate. Many of the cultures gave characteristic milk reaction of the alkali-forming group of bacteria.

TABLE 13.—*Fermentation of the sodium salt of citric and lactic acid together with the milk reactions of known cultures.*

Culture.	Litmus-milk reaction, 7 days at 30° C.	Pʜ value, 3 days at 37° C.		Culture.	Litmus-milk reaction, 7 days at 30° C.	Pʜ value, 3 days at 37° C.	
		Citric.	Lactic.			Citric.	Lactic.
Control		6.8	7.2	Dysentery—Continued.			
Proteus:				8	No change	7.4	7.3
ACL	Alkaline	8.4	6.9	9	Slight acid	7.4	7.1
ACN	do	8.8	8.6	10	No change	7.4	7.4
ACU	do	8.3	7.2	11	do	7.4	7.1
ACV	do	8.3	7.0	12	do	7.4	7.3
ADE	do	8.4	7.3	13	Coagulated, acid.	7.4	7.4
ADG	do	8.3	7.0	14	Slight acid	7.4	7.3
Typhoid:				15	do	7.4	7.4
607	Coagulated, acid.	7.5	7.3	16	Alkaline	7.4	7.5
608	No change	7.4	7.3	17	No change	7.4	7.4
Rowd	Alkaline	8.4	7.5	18	do	7.4	7.1
M. T. S	Slight acid	7.3	7.3	20	do	7.4	7.3
Hop	Coagulated, acid.	7.4	7.3	21	do	7.4	7.2
Hop 22	do	7.5	7.0	22	Coagulated, acd.	7.4	7.8
Paratyphoid:				23	Slight alkaline	8.6	Lost.
16	No change	7.4	7.0	24	Slight acid	7.4	7.0
22	Alkaline	8.5	7.3	25	do	7.4	7.0
323	do	8.5	7.7	26	do	7.4	7.0
B. enteriditis	do	8.5	7.3	27	No change	7.4	7.4
B. abortus	do	8.4	7.5	28	do	7.4	7.3
B. bronchisepticus.	do	7.3	7.3	29	do	7.4	7.3
Dysentery:				30	do	7.4	7.4
1	No change	7.4	7.3	31	do	7.4	7.3
2	do	7.4	7.1	32	Slight acid	7.4	7.1
3	Slight acid	7.4	7.3	33	No change	7.4	7.5
4	No change	7.4	7.3	34	do	7.4	7.3
5	Slight acid	7.4	7.4	35	do	7.4	7.3
6	do	7.4	7.4	36	do	7.4	7.3
7	No change	7.4	7.0	37	Slight acid	7.4	7.0
				38	No change	7.4	7.4

The salts of organic acids not only serve as a source of carbon for the alkali-forming bacteria and, therefore, are valuable test substances, but their use need not be limited to the bacteria of this group. In fact, it seems probable that they may have an extensive application in the study of other groups of bacteria.

REDUCTION OF NITRATES AND NITRITES.

In view of the fact that all the organisms of the alkali-forming group of bacteria could utilize nitrogen from nitrates, it is certain there must be a reducing action. It is of interest, therefore, to point out the action of these bacteria in reducing nitrates to nitrites, as is shown by the ordinary tests for reduction generally used in bacteriological work. The reduction of nitrate to nitrite was first tried in the ordinary nitrate broth, which consisted of 0.1 per cent peptone and 0.02 per cent potassium nitrate. Cultures were incubated in the broth for seven days at 30° C., then tested for the presence of nitrite. The results of these tests are shown in Table 14. It will be seen that according to this test only seven cultures reduced nitrate to nitrite. Reduction of nitrate was then tested by using a medium in which beef extract was the source of nitrogen. In one case 0.2 per cent extract was used with 0.02 per cent nitrate, and in another case 0.1 per cent extract with the same amount of nitrate. The results in the table show that after 24 hours in the 0.2 per cent beef extract 11 cultures reduced the nitrates, while in the 0.1 per cent beef extract 18 cultures gave a nitrite test. After seven days' incubation 47 cultures reduced nitrate in 0.2 per cent beef extract and 40 cultures in 0.1 per cent beef extract.

TABLE 14.—*Reduction of nitrates and nitrites in simple and complex media.*

Culture No.	0.1 per cent peptone, 0.02 per cent potassium nitrate.	0.2 per cent beef extract, 0.02 per cent potassium nitrate.		0.1 per cent beef extract, 0.02 per cent potassium nitrate.		Sodium nitrate, 0.2 per cent; sodium-lactate, 0.25 per cent; sodium-dibasic phosphate, 0.1 per cent.		Sodium nitrite, 0.2 per cent: sodium lactate, 0.25 per cent; sodium-dibasic phosphate, 0.1 per cent.	
	Nitrite test, 7 days.	Nitrite test, 24 hours.	Nitrite test, 7 days.	Nitrite test, 24 hours.	Nitrite test, 7 days.	Nitrite test, 14 days.	Change in titration.	Ammonia test, 14 days.	Change in titration.
Control	−	−	−	−	−	−	−0.05	−	−0.10
10	−	−	+	−	−	+	− .61	−	−0.72
12	−	−	+	−	−	+	− .93	−	− .84
31	−	−	−	−	−	−	− .15	−	− .59
49	−	−	+	−	−	+	− .93	−	− .76
59	−	−	−	−	−	+	−1.20	−	−1.28
60	−	−	+	−	+	+	−1.15	−	−1.08
61	−	−	−	−	−	+	−1.05	−	−1.21
62	−	−	−	−	−	+	−1.18	−	−1.31
66	−	−	+	+	+	+	−1.21	−	−1.16
72	−	−	+	−	+	+	− .58	−	− .66
73	+	+	+	+	+	+	0 [1]	−	− .42
101	−	−	+	−	+	+	− .95	−	− .99
102	−	−	−	−	−	+	− .95	−	− .95
103	−	−	−	−	+	0 [2]	0	−	0
104	−	−	+	+	+	+	− .84	−	− .87
105	−	−	+	−	+	+	− .92	−	−1.26
106	−	−	+	−	−	+	−1.12	−	− .86
107	−	−	−	−	−	+	− .82	−	− .49
108	−	−	+	+	+	+	− .89	−	− .90
111	−	−	+	−	+	+	−1.27	−	−1.05

[1] "0" means no change in titration. [2] "0" under nitrite test means no growth of culture.

TABLE 14.—*Reduction of nitrates and nitrites in simple and complex media*—Contd.

Culture No.	0.1 per cent peptone, 0.02 per cent potassium nitrate.	0.2 per cent beef extract, 0.02 per cent potassium nitrate.		0.1 per cent beef extract, 0.02 per cent potassium nitrate.		Sudium nitrate, 0.2 per cent; sodium-lactate, 0.25 per cent: sodium-dibasic phosphate, 0.1 per cent.		Sodium nitrite, 0.2 per cent: sodium lactate, 0.25 per cent; sodium-dibasic phosphate, 0.1 per cent.	
	Nitrite test, 7 days.	Nitrite test, 24 hours.	Nitrite test, 7 days.	Nitrite test, 24 hours.	Nitrite test, 7 days.	Nitrite test, 14 days.	Change in titration.	Ammonia test, 14 days.	Change in titration.
112	−	−	+	−	+	+	− .95	−	− .57
113	−	−	+	−	−	+	− .63	−	− .40
114	−	−	+	−	+	+	− .92	−	− .81
116	−	−	+	+	+	−	− .79	−	− .99
117	−	−	+	−	+	+	− .97	−	− .77
118	−	−	+	−	+	+	−1.07	−	−1.20
119	−	−	−	−	+	−	− .93	−	− .86
120	−	−	+	−	+	+	− .85	−	− .35
121	+	+	+	+	+	+	− .11	−	− .66
122	−	−	−	−	−	−	− .07	−	−1.40
123	−	−	+	+	−	+	− .97	−	− .63
124	−	−	+	−	+	+	− .87	−	− .99
125	−	−	+	+	+	+	−1.03	−	− .96
126	−	−	+	−	+	+	−1.01	−	− .84
128	−	−	+	−	+	+	− .85	−	− .85
130	−	−	−	−	−	+	−1.09	−	−1.12
131	−	−	−	−	−	−	− .97	−	− .68
132	−	−	−	−	−	+	− .93	−	− .91
133	+	+	+	+	+	+	− .05	−	− .62
134	+	−	+	+	+	+	− .91	−	− .51
135	−	−	−	−	−	+	−1.02	−	− .87
136	−	−	−	−	−	+	− .97	−	− .88
137	−	−	−	−	−	+	− .89	−	− .63
138	−	−	−	−	−	+	−1.15	−	−1.01
139	−	−	−	−	−	+	−1.10	−	−1.33
141	−	−	+	−	+	+	− .95	−	− .19
142	−	−	+	−	+	+	−1.08	−	−1.45
143	−	−	+	−	−	+	− .76	−	− .72
144	−	−	+	−	+	+	−1.07	−	−1.01
145	−	−	+	−	+	+	− .97	−	−1.34
146	−	−	+	−	+	+	−1.05	−	−1.00
147	−	−	−	−	−	0	0	−	−1.51
303	−	−	−	−	−	0	0	−	0
309	−	+	+	−	+	0	0	−	0
314	−	−	+	+	+	+	−1.19	−	−1.52
317	−	−	−	−	−	0	0	−	− .51
319	−	−	+	−	+	+	− .88	−	− .26
321	−	+	+	+	+	+	− .75	−	− .72
322	+	+	+	+	+	+	− .94	−	− .97
325	−	+	+	+	+	+	− .90	−	− .48
326	−	+	+	+	+	+	−1.00	−	− .51
340	−	+	+	−	+	0	0	−	0
353	+	+	+	+	+	+	− .41	−	− .23
354	−	−	−	−	−	+	− .84	−	− .61
356	+	+	+	+	+	+	−1.10	−	− .62
361	−	−	+	−	+	+	− .90	−	− .18
362	−	−	+	+	+	+	− .18	−	− .43

It is interesting to compare the number of positive nitrite tests in the peptone nitrate broth, where only seven cultures gave a positive nitrite test after seven days, with the 0.2 per cent beef-extract nitrate broth where 47 out of a total of 68 cultures showed a nitrite test after seven days. This small number of reductions

of nitrate to nitrite in peptone broth was probably due to the fact, which has been previously explained, that the organisms of the group did not develop well in peptone solution. While they could use it as a source of nitrogen with a suitable source of carbon, it was a poor source of nitrogen when used alone. With beef extract a source of carbon was probably supplied for the alkali-forming bacteria from the salts of the organic acid present in the extract; consequently these bacteria could grow better, and as a result there was a larger number of cultures which could reduce the nitrate to nitrite.

It has been seen, however, that these bacteria could utilize nitrogen from nitrate; consequently it should be expected that all the cultures should show a reduction and a positive nitrite test. Tests were made, therefore, in a medium containing 0.2 per cent of sodium nitrate, 0.25 per cent sodium lactate, and 0.1 per cent sodium-dibasic phosphate in distilled water. This medium was inoculated and incubated at 30° C. for 14 days and then tested for the presence of nitrite. Table 14 shows that 57 cultures gave a positive nitrite test. Seven did not grow in the experiment, although at other times they grew in the same medium. The remaining four cultures did not show a positive nitrite test. In order to show that there was a growth in all cases except the seven mentioned, titrations were made to determine the production of alkaline carbonates due to the oxidation of the sodium lactate. It will be seen from the table that all the cultures except those which did not show any growth produced an alkaline reaction. Even the five cultures which showed negative nitrite tests produced an alkaline reaction, showing that the nitrogen for growth must have been obtained from sodium nitrate; consequently there must have been a reduction. The probable reason why there was growth without a nitrite reaction is because nitrites may be utilized by this group of alkali-forming bacteria as well as nitrates. This is shown in Table 14 through the alkaline change in reaction in the medium composed of 0.2 per cent sodium nitrite, 0.25 per cent sodium lactate, and 0.1 per cent dibasic-sodium phosphate and distilled water.

It will be seen from the results that all but 5 cultures produced an alkaline change, showing the ability of the organisms to utilize sodium nitrite as a source of nitrogen and consequently reduce it. The five cultures that failed to show the nitrite test in the sodium-nitrate medium all produced an alkaline change in the sodium-nitrite-lactate medium. It is probable that these organisms failed to show a positive nitrite test because the nitrite was used up as soon as formed from the nitrate. Since these alkali-forming bacteria could reduce nitrite, it was natural to expect that it might be possible to determine the reaction by means of the determination of ammonia as shown by

Nessler's reagent; consequently all the cultures in sodium-nitrite-lactate medium were tested for the presence of ammonia. The test, however, was found to be negative and since it was known that these bacteria could utilize ammonia as a source of nitrogen the results indicate that as the nitrite was reduced to ammonia the latter was immediately used up.

The results showing the reduction of nitrate and nitrite by the alkali-forming group of bacteria are of particular interest, since they point out the difficulty of obtaining accurate tests for these reductions without a thorough understanding of the physiology of the bacteria.

ARBITRARY GROUPING OF THE ALKALI-FORMING BACTERIA FROM MILK.

An attempt has been made to arrange into groups the 68 alkali-forming bacteria used in this study in order to show the possibility and emphasize the use of the organic-acid salts, carbohydrates, and alcohols as test substances when used as the only source of carbon in a synthetic medium. In the chart, figure 2, it may be seen that the cultures have been divided first according to their morphology into cocci and bacilli, then further subdivided on the basis of their fermentation of dextrose. After the division of the cultures into positive and negative dextrose, they are again divided into subgroups according to their ability to ferment mucic, tartaric, malonic, or glyceric acid salt. In this grouping, in order to differentiate further and bring out the main points of difference in the cultures, the following organic salts were used—formic, butyric, valeric, hippuric, uric, and urea. While these organic-acid salts were used to group the cultures, it often happened that other easily fermented organic-acid salts not mentioned had to be utilized to distinguish the different subgroup members further. Examples of such are the salts of malic, acetic, and citric acids.

For the convenience of those who may study alkali-forming organisms each subgroup has been given an arbitrary number. The fermentation of dextrose and the salts of the organic-acid salts mentioned were, for the purpose of this paper, considered primary characteristics in distinction to the secondary characteristics which are shown in the lower half of figure 2. It is of interest to observe that the alkali-forming bacteria studied in this work and grouped according to what are considered primary characteristics, also had secondary characteristics which correlated with the primary. Thus by referring to the chart it may be seen that all the fluorescent cultures fell into subgroup 4, all the gelatine liquefiers fell into subgroup 6, while all the cultures that fermented the alcohols, such as mannite and glycerin, fell into subgroup 9. The only cultures that showed gas in dextrose broth also were in subgroup 9. The pigment formers, which were also the cultures that fermented saccharose, fell into

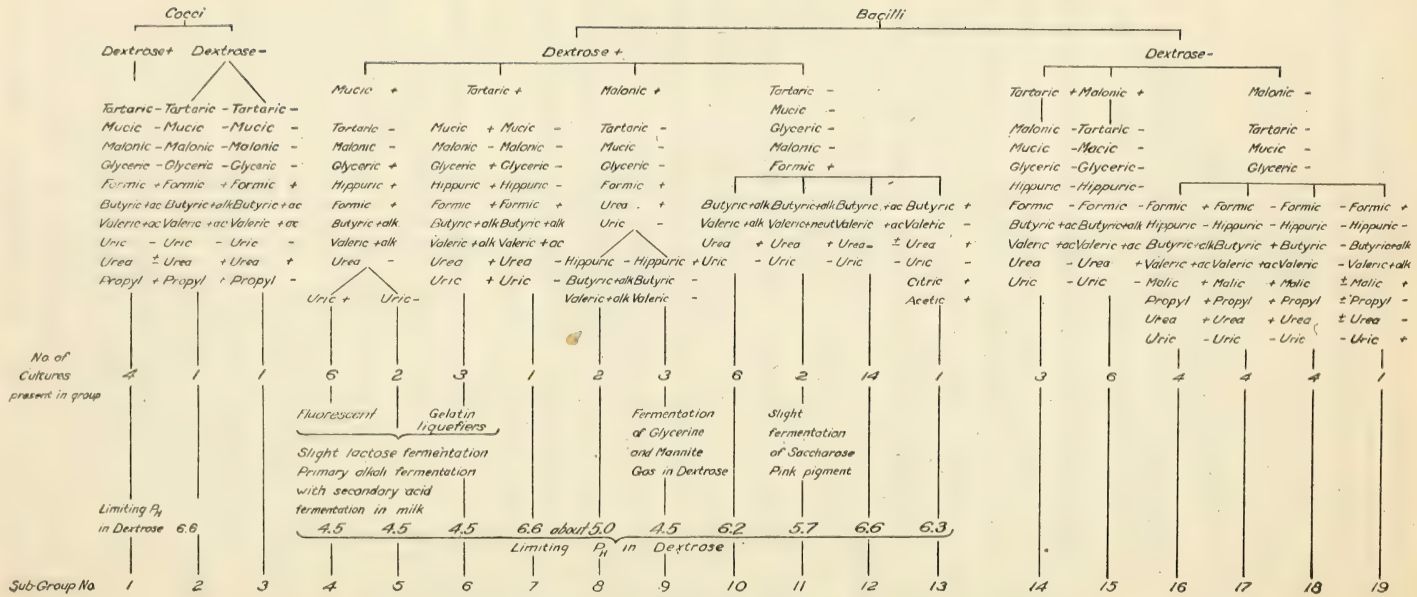


FIG. 2.—Arbitrary grouping of the alkali-forming bacteria studied in this investigation.

subgroup 11. Another noticeable secondary characteristic was the uniform limiting P_H value in dextrose medium A, the composition of which was given on page 13. Each subgroup of the dextrose fermenters utilized the carbohydrate to a practically constant limiting hydrogen-ion concentration, as is shown for example in subgroup 10, where all the 6 cultures fermented dextrose to P_H 6.2 after 7 days' incubation at 30° C. The reaction in milk was similar in subgroups 4, 5, and 6. In these 3 groups there was first a primary alkali fermentation which was followed by what was apparently a secondary acid fermentation, during a period of 30 days' incubation. The secondary characteristics of this group have been given in detail so as to emphasize further and show the possibility of the new method of grouping alkali-forming bacteria according to their ability to utilize carbon when supplied in a synthetic medium from organic-acid salts, carbohydrates, and alcohols. It was impossible to make this study cover all types of the alkali-forming bacteria, because the definition chosen for this group is so simple and yet so comprehensive that it must include a very large number of species of bacteria. Therefore, the scheme for grouping this particular collection of alkali-forming organism is not intended for a definite classification of all the alkali-forming group of bacteria. It should serve, however, as a possible basis for future work along this line and particularly to draw attention to the fact that the fermentation of organic-acid salts is a reliable and important means for classification, especially for organisms of this type occurring in soil.

SUMMARY AND CONCLUSIONS.

1. The alkali-forming bacteria may be defined as those bacteria which produced an alkaline reaction in milk without visible signs of peptonization. In litmus milk this reaction appeared sometimes within 48 hours, usually within 5 days, while occasionally a longer period was required. The alkaline reactions were caused primarily by the oxidation of the salts of citric acid to alkaline carbonates. Other organic-acid salts if present in milk may also be similarly fermented. Ammonia played little or no part in causing the alkaline reaction during the first 7 days of incubation, but at later periods a few cultures produced considerable ammonia. Several cultures showed a primary alkaline fermentation which was followed by what was probably a secondary acid fermentation.

2. The alkali-forming bacteria studied were isolated principally from milk, a few being obtained from ice cream. These organisms were found in large numbers in numerous samples of soil and also water. They were present in large numbers on the hands of farm laborers, and in unsterilized milk utensils. While not present in the udders of cows, the alkali-forming bacteria were present in relatively small numbers in cow feces. It seems evident that the

organisms of this group found in milk were principally soil bacteria introduced into milk largely from unsterilized utensils, with dust, or from the dirty hands of milkers. Cow feces may also play a small part in the contamination. In general, soil can not be considered the only source of these organisms, because there are many bacteria from other sources, which would be placed in this group on account of producing the characteristic milk reaction.

3. Cultures of the bacteria included both cocci and bacilli which grew best aerobically at temperatures ranging from 20° to 30° C. When heated in milk for 30 minutes, the thermal death point of these organisms ranged from 60° to 65.6° C. No spores were observed. Most of the cultures produced a slimy growth on agar, several showed fluorescence and a few showed pigment formation.

4. Sodium-ammonium phosphate was used as a source of nitrogen in the study of the alkali-forming bacteria. While the organisms could utilize nitrogen from numerous organic materials when suitable sources of carbon were supplied, either contained in them or supplied from other sources, it was found necessary in studying the fermentation of test substances to have a definite source of nitrogen free from carbon.

5. In studying the fermentation of test substances, nitrogen was supplied by sodium-ammonium phosphate, while the test substance furnished the carbon. The ordinary beef-extract broth was not found to be suitable for the determination of the fermentation of carbohydrates, because carbon was available either from the carbohydrate used or the organic-acid salts present in the beef-extract broth. It was possible, therefore, for a simultaneous acid (from the carbohydrate) and alkaline (from the organic-acid salts) fermentation to take place, in which case the final reaction would depend on the rate of the two fermentations. It was often found that in beef-extract broth the acid fermentation was entirely masked by the alkali fermentation. To overcome this as far as possible a synthetic medium was used which contained a single source of nitrogen and single but different source of carbon. Dextrose was the most easily fermented of the carbohydrates tested. Of the 68 cultures examined 44 fermented dextrose, 11 lactose, and 2 saccharose, while none fermented raffinose. Dextrose and galactose were fermented to about the same extent and many media showed a high hydrogen-ion concentration, while lactose and saccharose were fermented only slightly. In extract broth there was no evidence of a lactose fermentation—in fact, the reaction went alkaline—but in the synthetic medium a sight but distinct fermentation was observed.

6. The monoatomic alcohols ethyl, propyl, and amyl were more readily fermented than the polyatomic alcohols mannite and glycerin. The alcohols tested were the only source of carbon in the sodium-ammonium-phosphate medium.

7. Organic-acid salts were easily available as sources of carbon when used in the synthetic medium, and in most cases an alkaline reaction was produced, due to the formation of bicarbonates or carbonates. The exceptions were when the salts of n–butyric, n–valeric, or caproic acids were used. These acids may be split into other acids and if the fermentation is not carried far enough the reaction may become acid.

An attempt was made to determine from what radical or radicals the alkali-forming bacteria obtained their carbon. The results of the study indicate that it may come from the methyl, alcohol, or aldehyd (as in formic acid), but not from the carboxyl group (as in oxalic acid). Carbon was most easily obtained from the methyl group (as in succinic, acetic, etc.), and about as well from the methyl and alcohol group when attached (as in citric, malic, or lactic acid). Carbon was not easily available from the alcohol radical when it was linked to a carboxyl and another alcohol radical (as in mucic, tartaric, and glyceric acids). It was not available from an alcohol radical when linked to a carboxyl (as in glycolic acid). Apparently the benzol ring can be split by some bacteria, for a few of the cultures were able to obtain their carbon from the salts of benzoic and salicylic acids.

Some of the cultures were able to obtain both nitrogen and carbon from urea and also from uric and hippuric acids. Numerous known cultures, some of which give the characteristic alkaline milk reaction of the alkali-forming group, were capable of utilizing the salts of organic acids as a source of carbon when grown in a synthetic medium. Among these were cultures of proteus, typhoid, paratyphoid, and dysentery; also *B. abortus*, *B. bronchisepticus*, *B. fæcalis alkaligenes*, and *B. enteriditis*. It is believed therefore that the salts of organic acids may serve as valuable carbon-containing test substances for the further identification of bacteria.

8. The alkali-forming bacteria readily used the nitrates and nitrites as sources of nitrogen, but when tested for the reduction of nitrates and nitrites in the commonly used peptone medium very few showed any evidence of reduction. The ordinary method of testing the reduction of nitrates to nitrites is of no value when alkali-forming bacteria are being studied. This may be equally true of other groups of bacteria. Special media should be used, therefore, whenever possible.

9. An arbitrary division of the alkali-forming bacteria found in milk has been made. This classification was based primarily on the fermentation of dextrose and the salts of organic acids, and serves to show the value of the fermentation of the organic-acid salts for this purpose. Each subgroup has been given a number as a matter of convenience for those who may study these types of bacteria in

the future. No attempt has been made to group these cultures on the basis of the accepted principles of classification.

10. It is hoped that the results presented may give a clearer conception of certain types of alkaline fermentation. Probably many alkaline reactions presumed to be due to ammonia formation are in reality the result of the oxidation of organic-acid salts to alkaline carbonates. These alkaline fermentations, occurring as they may with acid fermentations, greatly complicate the fermentation of various test substances. This type of alkaline fermentation may explain many obscure results which occur in bacteriological studies. Furthermore, since organic acids are suitable sources of carbon for many bacteria, they may serve as an extensive set of test substances by means of which the physiological characteristics of bacteria may be further studied. The fermentation of organic-acid salts should be of particular value in the study of bacteria which fail to ferment the usual test substances, such as carbohydrates, alcohols, and starches.

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